# THE GENETICS OF PSORIATIC ARTHRITIS

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# The Genetics of Psoriatic Arthritis

by

© Christopher D. Butt, M.Sc.

A thesis submitted to the School of Graduate Studies

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# Abstract

Psoriasis is a stubborn chronic skin disease affecting 1-3% of the population. Although not life threatening, it may be associated with important morbidity and disability. Psoriatic Arthritis (PsA), an immunologically mediated disease, is an inflammatory form of arthritis usually seronegative for rheumatoid factor, which may affect as many as 30% of patients with psoriasis, thus up to 1% of the population may have PsA. Whereas the prevalence of inflammatory arthritis in the general population is estimated at 2 to 3%, in patients with psoriasis the prevalence of inflammatory arthritis varies from 6 to 42%. PsA is highly heritable with the risk ratio for siblings of PsA patients estimated at 30.8 times that of the general population. PsA is T-cell driven disorder and the pathogenesis derives from multiple processes including synovial and entheseal inflammation, angiogenesis, and altered bone remodelling.

Association studies have repeatedly implicated the *HLA-Cw*0602* loci of the Major Histocompatibility Complex (MHC) in the aetiology of PsA. Numerous other genes from several pathways have also been implicated in PsA. Using the unique population resource of Newfoundland, and a validation cohort from Toronto, it has been observed that there is an association between PsA and the MHC genes *TNF-α* and *MICA*. For the first time, an association has also been observed between PsA and the pro-angiogenic genes *VEGF* and *PPARγ*. An association was also observed with the pleiotropic autoimmune gene *PTPN22*, and for the first time epistatic gene-gene interactions have been observed in PsA via a novel algorithm, adding further evidence to the central involvement of *IL-23R* in PsA.

# Acknowledgements

Once again, and as always, this is for Gordon Saunders. Thank you for everything all those years ago. None of this could have happened without you. Opening move: King's pawn to King 4 – your turn. I hope we get to play another game someday soon.

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## List of Abbreviations

| | |
|---|---|
| AS | Ankylosing Spondylitis |
| CASPAR | Classification of Psoriatic Arthritis |
| CCA | Competitive Co-evolutionary Algorithm |
| CD | Crohn disease |
| CDSN | Corneodesmosin |
| DIP | Distal Interphalangeal |
| DMARD | Disease-modifying anti-rheumatic drugs |
| DNA | Deoxyribose nucleic acid |
| EGF | Epidermal growth factor |
| FBAT | Family Based Association Test |
| FGF | Fibroblast growth factor |
| GRR | Genotype Relative Risk |
| GWAS | Genome Wide Association Study |
| HLA | Human Leukocyte Antigen |
| HRR | Haplotype Risk Ratio |
| HWE | Hardy-Weinberg Equilibrium |
| IBD | Inflammatory bowel disease |
| IDDM | Insulin-dependent diabetes mellitus |
| IL | Interleukin |
| LD | Linkage disequilibrium |
| MHC | Major Histocompatibility Complex |
| MICA | Major histocompatibility complex classI-related gene A |
| NFKB1 | Nuclear-factor kappa-beta 1 |
| NSAID | Non-steroidal anti-inflammatory drugs |
| OPC | Osteoclast precursor cells |
| OPG | Osteoprotegerin |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| PPARG | Peroxisome proliferator activated receptor-gamma |
| PsA | Psoriatic Arthrits |
| PTPN22 | Protein tyrosine phospatase non-receptor type 22 |
| RA | Rheumatoid Arthritis |
| RANK | Receptor activator of nuclear factor kappa B |
| RANKL | Receptor activator of nuclear factor kappa B ligand |
| RNA | Ribose nucleic acid |
| SEC | Synovio-entheseal complex |
| SLE | Systemic lupus erythematosus |
| SNP | Single Nucleotide Polymorphism |
| SpA | Undifferentiated spondyloarthropathy |
| TDT | Transmission disequilibrium test |
| TNF | Tumour Necrosis Factor |
| UC | Ulcerative colitis |
| VEGF | Vascular endothelial growth factor |

# Co-authorship Statement

This thesis consists of nine published articles, one article which is an expanded version of a published abstract, and three others articles which we intend to submit for publication. Of these 13 articles, I am listed as primary author on 10. For any article in which I am listed as primary author, I participated in study design, performed the molecular genetic laboratory work, assisted in co-ordinating the statistical analysis, and drafted the original manuscript.

I am listed as second author for chapter 3.4 "Association of *SEEK1* and psoriatic arthritis in two distinct Canadian populations" and for Chapter 6 "Epistasis: a novel algorithm detects gene-gene interaction in psoriatic arthritis". For Chapter 3.4 I performed the molecular genetic laboratory work and assisted in editing the original manuscript. For Chapter 6, I co-conceived of the study design, I performed the molecular genetic laboratory work, and co-authored the manuscript. As this chapter is largely dependent on an algorithm, I feel it is appropriate that Mr. Mohammed Uddin receive primary author credit, as the algorithm (which is included in Appendix 1) is entirely his design.

I am listed as third author for chapter 3.1 "TNF-$\alpha$ Polymorphisms and Risk of Psoriatic Arthritis in Caucasian Populations". For this article, I performed the molecular genetic laboratory work on the Newfoundland cohort, and assisted with editing of the original manuscript.

# 1

# INTRODUCTION

## 1.1 – The Phenotype of Psoriatic Arthritis

Psoriasis and psoriatic arthritis (PsA) are interrelated disorders, as most PsA patients have psoriasis (1). Psoriasis is a stubborn, chronic, T-cell mediated inflammatory skin disease affecting 1-2% of the Caucasian population (2), and 2-3% of the population worldwide (3). The differing population prevalences of PsA are given in Table 1-1. Although not life threatening, PsA may be associated with important morbidity and disability. An association between inflammatory arthritis and psoriasis, termed PsA, was formally recognized by the American Rheumatology association in 1964 (4), but was probably first described by Alibert in 1818 (5). PsA is an inflammatory form of arthritis usually seronegative for rheumatoid factor (6), which may affect as many as 30% of patients with psoriasis, thus up to 1% of the population may have PsA (7,8). PsA runs a highly variable course, from a mild non-destructive disease to a severe rapidly progressive erosive arthropathy (9).

The existence of PsA as a distinct clinical entity remains a topic of debate; some authors propose that it is simply the co-occurrence of psoriasis and inflammatory arthritis. As recently as July 2007, an editorial by Maxime Dougados entitled "Psoriatic Arthritis: Is it for Real?" was published in Joint Bone Spine (10). A disease entity, if real is likely to have a distinct prevalence differing from the expected co-occurrence of psoriasis and inflammatory arthritis and different susceptibility factors in addition to those that contribute to psoriasis and inflammatory arthritis alone.

**Table 1-1: Prevalence of PsA as reported in studies of different populations**

| Study/Year | Country | Prevalence (%) |
|---|---|---|
| Lomholt, 1963 | Faroe Islands | 0.04 |
| Hellgren, 1969 | Sweden | 0.02 |
| van Romunde, 1984 | Netherlands | 0.05 |
| Shbeeb, 2000 | USA | 0.1 |
| Alamanos, 2003 | Greece | 0.06 |
| Trontzas, 2005 | Greece | 0.17 |
| Madland, 2005 | Norway | 0.2 |
| Gelfand, 2005 | USA | 0.25 |

*Adapted from* (11)

## 1.2 - Epidemiology of Psoriatic Arthritis

Psoriasis exists in either an early onset, prior to age 40, typically familial form (Type I), or as a later onset, more sporadic disease (Type II). Psoriasis typically precedes the onset of arthritis in roughly 70% of PsA patients, but may appear after (20%) or at the same time as (10%) as the development of arthritis (12). Estimates of the prevalence of PsA have been given as roughly 0.1% in Rochester Minnesota (13) to 2.5% in Toronto (14), to 0.04% to 1.2% in Sweden (15-17), while Gelfand et al. reported in 2005 a PsA prevalence of 0.25% (95% CI : 0.18%, 0.31%) (18). A recent survey by the National Psoriasis Foundation suggests prevalence of PsA to be 1.4% of general population in the US and

anywhere from 6-42% in psoriasis patients (19), and a recent European study that utilized a rheumatologist and dermatologist estimated a prevalence of inflammatory arthritis in psoriasis to be 30% (20). The prevalence of PsA in psoriasis patients from different populations is given in table 1-2. Given that the prevalence of inflammatory arthritis in the general population is estimated at 2 to 3% (3), the increased prevalence of PsA in psoriasis patients argues for a distinct disease entity. As an interesting corollary, it appears that psoriasis occurs more often in patients with a background of inflammatory arthritis. Harrison *et al.* (21) found that 5.3% of patients with early inflammatory polyarthritis had psoriasis on examination. Although this prevalence is thought to be higher than the general prevalence of psoriasis among Caucasians (roughly 1 – 2% (3,22)), Harrison *et al.'s* study did not include and internal control group for direct comparison. The evidence shows overwhelmingly that psoriasis and PsA are interrelated disorders.

## 1.3 - Clinical Features of Psoriatic Arthritis

The descriptions of arthritis associated with psoriasis complement the argument that it is a distinct entity. Unlike osteoarthritis, PsA is inflammatory in nature, and affects both proximal and distal joints. It differs from Rheumatoid arthritis (RA) by the lack of gender preference, the frequent involvement of distal interphalangeal

**Table 1-2: Prevalence of PsA as reported in psoriasis patients from different populations**

| Study | Centre | Patients with Psoriasis | PsA (%) |
|---|---|---|---|
| Leczinsky, 1948 | Sweden | 534 | 7 |
| Vilanova, 1951 | Barcelona | 214 | 25 |
| Little, 1975 | Toronto | 100 | 32 |
| Scarpa, 1984 | Naples | 180 | 34 |
| Stern, 1985 | Boston | 1285 | 20 |
| Zaneli, 1992 | Winston-Salem, USA | 459 | 17 |
| Barisic-Drusko, 1994 | Osijek region | 553 | 10 |
| Salvarani, 1995 | Reggio Emilia | 205 | 36 |
| Shbeeb, 2000 | Mayo Clinic, USA | 1056 | 6.25 |
| Brockbank, 2001 | Toronto | 126 | 31 |
| Alenius, 2002 | Sweden | 276 | 48 |
| National Psoriasis Foundation, 2002 | USA | 4.4 male | 23 |
| Zachariae, 2003 | Denmark | 5795 | 30 |

*Adapted from* (11)

(DIP) joints, the tendency of asymmetry, the absence of rheumatoid factor, the presence of spondyloarthropathy, as well as associations with HLA-B27 and the presence of extra-articular features common to the spondyloarthropathies (23). No laboratory diagnostic test exists for PsA. The erythrocyte sedimentation rate is elevated in 40 to 60% of PsA patients, particularly in those with the polyarticular form (8). In the past, the lack of rheumatoid factor activity was considered the

most distinctive laboratory feature of PsA. Studies have indicated, however, that low titres of rheumatoid factor are detected in 5-16% percent of patients (6) (24) (23).

The majority of PsA patients have the classic form of psoriasis vulgaris, although pustular psoriasis and erythroderma have also been reported, and in approximately 70% of cases psoriasis precedes the onset of arthritis (25). The type of psoriasis itself may play a role in determining the onset of PsA. A study examining the onset of PsA determined that patients with Type I psoriasis tend to develop skin disease approximately 9 years before joint disease, while those with Type II psoriasis tend to display skin and joint manifestations within a year of each other (26). There appears to be no clear relationship between the extent of psoriasis and severity of inflammatory arthritis (27). Nail lesions are the only clinical feature of skin psoriasis that is significantly associated with the development of PsA. Indeed, only 35 percent of patients recognize a link between the joint and skin disease (8). The onset of arthritis is usually slow and begins mildly before progressing, but it can occur acutely (23), and the course of PsA is usually characterized by flares and remissions. The first classifications of PsA based on arthritic clinical patterns were defined by Moll and Wright (6), and broke the disease into 5 patterns: 1) So called 'Classic PsA' confined to DIP joints of the hands and feet, affecting approximately 5% of PsA patients; 2) Arthritis Mutilans (i.e., bone resorption), otherwise known as destructive arthritis, often with sacroiliitis, affecting less than 5% of patients; 3) Symmetric polyarthritis indistinguishable from RA, which accounts for roughly 50% of all PsA cases; 4)

Asymmetric oligoarthritis affecting roughly 35% of patients, and finally; 5) Spondyloarthropathy, affecting 20-40% of patients and usually in association with peripheral joint involvement. Figure 1-1 (a – g) shows the physical manifestations of the different types of PsA in patients. Subsequent reports validated these subgroups; however there is considerable overlap and evolution between them, and the disease patterns can occur in combination (28). In addition, variability in the reporting physician's definition of symmetry, symmetric oligoarthritis, and peripheral and axial overlap pattern has led to differences in the reported frequency of the subsets of PsA (23).

Spondylitis is rarely observed at the onset of PsA: it tends to effect men and older patients, and it tends to begin later in the course of the disease (29). The spondylitis can include sacroiliitis (often asymmetric) and spinal disease similar to AS consisting of pain and stiffness of the cervical, thoracic and lumbar spine. Other clinical features of PsA include dactylitis and enthesitis (6,30,31). Dactylitis occurs in more than 30% of patients and is characterized by a diffuse swelling of the entire digit along with arthritis of the distal and proximal interphalangeal and metacarpophalangeal or metatasrophalangeal joints. Magnetic resonance imaging studies have revealed effusions in the affected joints and within tendon sheaths (32). Further it is clear that patterns of PsA are not permanent; more than 60% change from their initial disease pattern (1,33). McHugh *et al.* (34) observed this phenomenon of changing disease patterns in a five year prospective study in which they followed 87 consecutive PsA patients. They found that eighteen patients changed subgroup; 11 had an increase

**Figure 1-1(a-g): Photographs and X-rays of the various clinical patterns of Psoriatic Arthritis**

Fig 1-1a: Oligoarthritis

Fig 1-1b: Distal arthritis

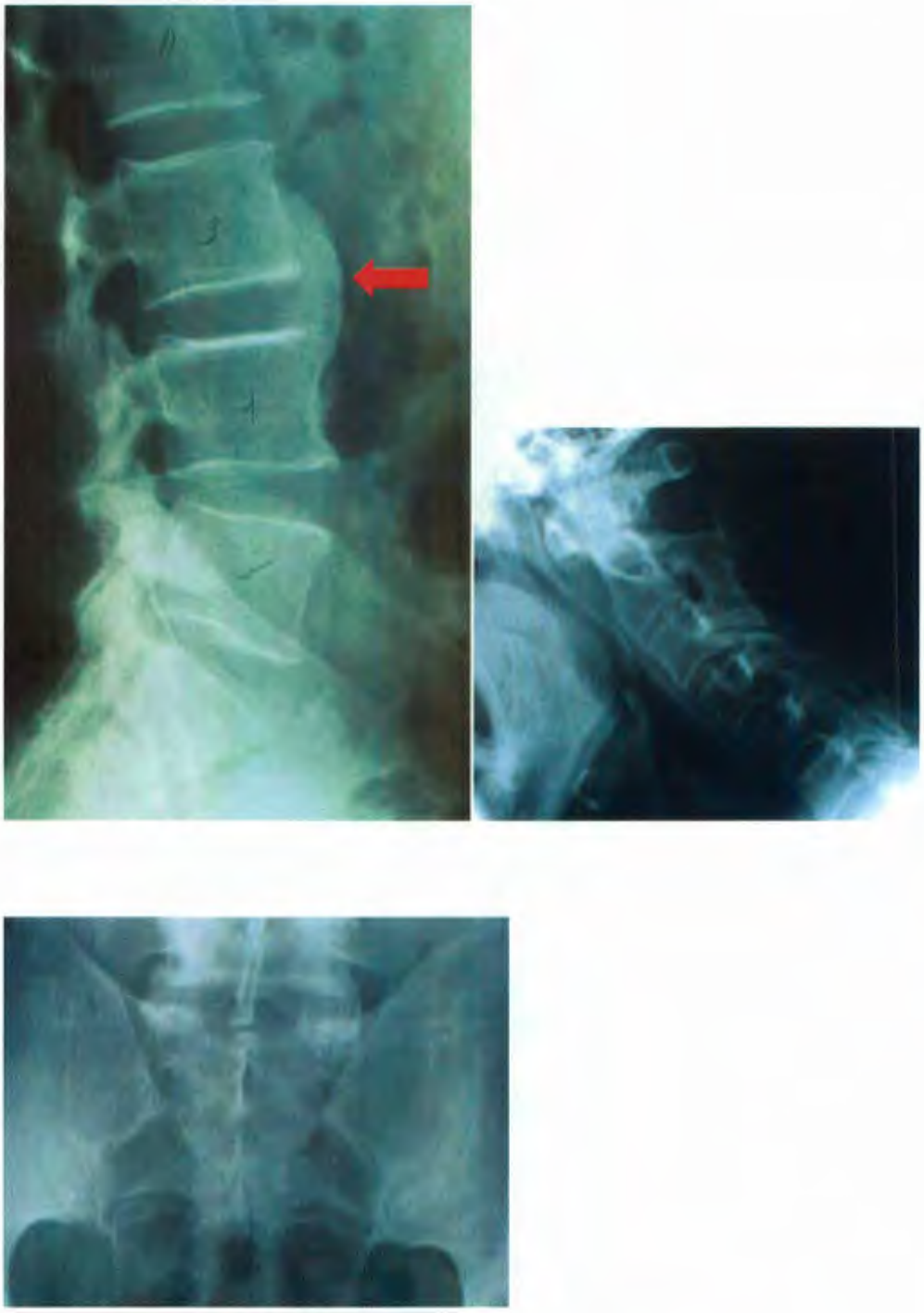

Fig 1-1c: Psoriatic Spondyloarthropathy

Fig 1-1d: Polyarticular arthritis

Fig 1-1e: Arthritis Mutilans

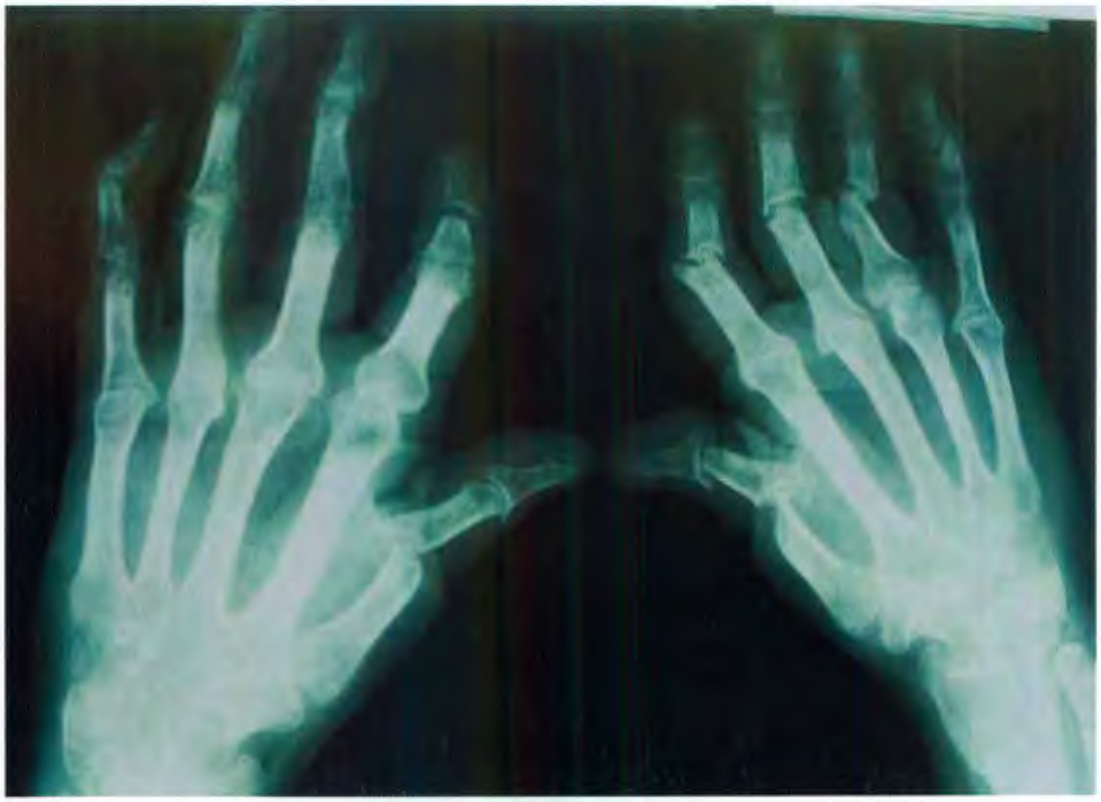

Fig 1-1f: Hand X-ray of severe Psoriatic Arthritis

Fig 1-1g: Foot X-ray and photo of severe Psoriatic Arthritis

in the number of joints involved, six a decrease, and one changed from an oligoarticular pattern to predominant spondylitis. Within the polyarticular group 37/51 patients had an increase in the number of joints involved. For the whole population, there were significant increases in the number of joints involved. There were no significant differences in skin and nail scores although nine more patients had developed nail disease.

Despite attempts to optimize medical therapy, a large number of patients with PsA go on to develop erosive polyarthritis while some patients develop ankylosis. The prevalence of deformity and damage in PsA is similar to that reported in RA (23). Over time, there is clinically active arthritis such that by the time patients have been followed for more than 10 years, 55% have five or more deformed joints (29). One study of patients with the early onset form of PsA showed that 47% presented to the clinic with at least one joint erosion within two years of disease onset (35). Thus PsA can be a destructive, inflammatory arthritis resulting in a significant health and economic burden (36).

Likely owing to the debate regarding the acceptance of the existence of PsA as a distinct disease entity, until recently it lacked a widely-agreed upon or validated case definition (37). While the Moll and Wright classification criteria for PsA has been in use for many years, the heterogeneity of clinical features of the disease has resulted in several different attempts to further refine the classifications for the disease (8,38-42). Recently, the CASPAR study group (**C**l**AS**sification of **P**soriatic **Ar**thritis*)* compared the test performance characteristics of these criteria using data retrospectively obtained from case

notes and radiographs of patients with PsA or RA, in order to determine more accurate and acceptable diagnostic criteria for PsA (25). Data were collected on 588 PsA cases and 536 controls with RA (n=384), ankylosing spondylitis (AS) (n=72), undifferentiated arthritis (n=38), connective tissue disorders (n=14), and other diseases (n=28). The CASPAR diagnostic criteria for PsA consists of established inflammatory articular disease (joint, spine, or entheseal) with at least 3 points from the following five categories: 1) [A] Current psoriasis (assigned a score of 2; all other features were assigned a score of 1); [B] A history of psoriasis (unless current psoriasis was present); [C] A family history of psoriasis in a first or second degree relative. 2) Nail dystrophy; 3) Seronegativity for rheumatoid factor; 4) Dactylitis; 5) Radiographic evidence of juxtaarticular new bone formation. When applied to PsA patients presenting at rheumatology clinics, these criteria were found to be more specific, but slightly less sensitive than the various existing diagnostic criteria in use. One major purpose of studies for these classification criteria is to enroll appropriate patients into clinical intervention, thus criteria with high specificity are more desirable than criteria with high sensitivity. The CASPAR criteria have been derived from observed patient data, are more specific in a rheumatology clinic setting, and are easier to use than existing classification criteria. Thus, the CASPAR criteria (Summarized in Table 1-3) have rapidly become the gold standard for PsA diagnosis.

Even with agreed diagnostic criteria, there remains disagreement as to the severity of the disease. While the disease patterns described by Moll & Wright and CASPAR may be clearly recognized at disease onset, it has been difficult to

**Table 1-3: CASPAR Criteria for diagnosis of PsA**

| Inflammatory musculoskeletal disease (joint, spine, or entheseal)<br>With **3** or more of the following: | | |
|---|---|---|
| 1. Evidence of psoriasis (one of a, b, c) | a. Current psoriasis* | *Psoriatic skin or scalp disease present today as judged by a dermatologist or rheumatologist* |
| | b. Personal history of psoriasis | *A history of psoriasis that may be obtained from patient, family doctor, dermatologist or rheumatologist* |
| | c. Family history of psoriasis | *A history of psoriasis in a first or second degree relative according to patient report* |
| 2. Psoriatic nail dystrophy | | *Typical psoriatic nail dystrophy including onycholysis, pitting and hyperkeratosis observed on current physical examination* |
| 3. A negative test for rheumatoid factor | | *By any method except latex but preferably by ELISA or nephelometry, according to the local laboratory reference range* |
| 4. Dactylitis (either a or b) | a. Current Dactylitis | *Swelling of an entire digit* |
| | b. History of Dactylitis | *Recorded by a rheumatologist* |
| 5. Radiological evidence of juxta-articular new bone formation | | *Ill-defined ossification near joint margins (but excluding osteophyte formation) on plain x-rays of hand or foot* |

**Specificity 98.7%, sensitivity 91.4%. *Current psoriasis scores 2**

*Adapted from Taylor W, et al. 2006*

ascertain whether these patterns have prognostic implications since there have been few prospective longitudinal studies in this disease. Moreover, in the few studies that did follow patients over time, the patterns demonstrate change over time (1,28,33). Earlier studies suggested that PsA was not a severe disease, but more recent experience describes a less benign clinical course, with the majority of patients (60%) presenting with polyarthritis (8,43), and with evidence of progression of deformities over time in a large proportion of patients (8). As mentioned previously, psoriasis precedes the onset of arthritis in approximately 70% of cases. The interval between the onset of psoriasis and arthritis can be extremely variable. There is no direct correlation between the severity of skin and joint manifestations except perhaps in those patients with simultaneous onset of both manifestations (39,44,45). Nail lesions, including pits and onycholysis signal the development of PsA (46), but at the same time, the presence of nail lesions appears to modify the risk for mortality in this disease (47). Nail lesions occur in 90 percent of patients with PsA and in only 41 percent of those with psoriasis uncomplicated by arthritis (46).

Gladman *et al.* introduced the concept of severity in psoriatic arthritis in 1987 (8), while others have recently confirmed that patients with psoriatic arthritis have a severe, progressive disease (34,43,48). Gladman *et al.* further demonstrated that over a 5 year period there is progression of both clinical and radiological damage in PsA, despite medical therapy (49,50), and that the radiological changes of patients with PsA are similar to those of patients with RA

(51), indicating that while the two diseases are distinct medical conditions, some underlying mechanisms in the progression of the diseases may be similar. They further demonstrated that progression of clinical damage was related to the number of actively inflamed joints at presentation (30). The predictive role of polyarthritis in the development of further damage was recently confirmed in another cohort of patients with PsA (46), while further studies also demonstrated that persistent inflammation leads to further damage (52). Gladman *et al.* have also demonstrated that disease severity is associated with early mortality in patients with PsA (47) (36). On the other hand, there are patients who may have milder disease course and experience remission (53).

With respect to mortality, one study indicated that there was no significant increase when comparing PsA patients to the general population (13). Studies from hospital environments, however, have indicated that there is an increased risk of death for PsA patients, with a standardized mortality ratio of 1.59 (female) – 1.65 (male) (36). Disease severity also appears to predispose for increased mortality (47), however, more recent reports indicate that there has been improved survival rates among PsA patients with the advent of newer therapies. Follow up data indicates that the mortality risk has dropped, particularly for males, to an overall mortality risk of 1.36. Of interest, the numbers of years of life lost was calculated at 3 years, similar to the number reported for patients with severe psoriasis (54).

## 1.4 Co-morbidities of Psoriatic Arthritis

Psoriasis and PsA are both T cell driven diseases (55,56) (this will be discussed in greater detail in section 1.6). A specific T cell subset known as T helper 1 (Th1) cells are responsible for an increase in antigen presentation and T cell activation. Increasing evidence suggests that a Th1 immune response, including activated T cells, antigen presenting cells, cytokines, and markers of systemic inflammation such as C-reactive protein, are important factors in the development of atherosclerotic plaques and ultimately, myocardial infarction (57). The observation that other Th1 driven disease such as RA have an increased risk of myocardial infarction adds support to the hypothesis that Th1 mediated disease may predispose to cardiovascular disease (58,59). The excess risk of cardiovascular disease in psoriasis has been demonstrated in a number of observational studies (60-64). One clinic-based study found that the risk of arterial and venous vascular diseases (e.g., myocardial infarction, thrombophlebitis, pulmonary embolization, and cerebrovascular accident) was 2.2 times higher among patients with psoriasis compared with control patients with other dermatologic conditions (61). In a large European referral cohort of patients with psoriasis, obesity, diabetes mellitus, heart failure, and hypertension were almost twice as frequent in patients with psoriasis as in control subjects (62). One recent study looked at 127,139 mild and 3837 severe psoriasis patients and 556,995 control patients and found that - after adjusting for hypertension, diabetes, history of myocardial infarction, hyperlipidemia, age, sex, smoking, and

body mass index - for a 30-year-old patient with mild or severe psoriasis, the adjusted RR of having an MI is 1.29 (95% CI, 1.14 - 1.46) and 3.10 (95% CI, 1.98 - 4.86), respectively, and concluded that psoriasis likely confers an independent risk for myocardial infarction (64).

While obesity and the often accompanying metabolic syndrome are also risk factors for MI, they are serious medical conditions of their own accord often requiring medical intervention. Several reports have shown an increased rate of obesity in case control studies (65-67). Metabolic syndrome, which comprises a cluster of risk factors, including obesity, dyslipidaemia, hypertension and glucose intolerance which can lead to diabetes, is a strong predictor of cardiovascular disease. Two case–control studies have demonstrated an increased prevalence of metabolic syndrome and its contributing components in patients hospitalized for severe psoriasis (66,68).

While psoriasis and PsA are interrelated disorders, there is much less literature available regarding the accompanying co-morbidities of PsA. One recent review reported on the varying causes of death amongst PsA patients, and found that between 25% - 55% of PsA patients die from cardiovascular disease, between 10% - 25% from respiratory illnesses, and 11% - 24% die from cancer (69). It becomes apparent when examining the co-morbidities that are associated with psoriasis and PsA, that several different pathological mechanisms must be involved in these disease processes.

## 1.5 - Treatment of Psoriatic Arthritis

Until very recently, PsA was considered to be a very mild disease, and patients were usually treated with non-steroidal anti-inflammatory drugs (NSAIDs) (9). In the usual manner of treatment during the early stages of disease, the physician typically attempts to treat the most prominent issue for the patient. If the major issue is the skin manifestations, then they are treated first, usually by a dermatologist, and the joint manifestations are managed with NSAIDs (27). NSAIDs, however, are associated with significant adverse events, and it must be noted that they do not modify the course of the disease, nor do they prevent development or progression of erosions. Thus, if patients respond poorly to NSAIDs or if there is already erosive disease, patients should be treated aggressively with disease-modifying anti-rheumatic drugs (DMARDs) – although ideally patients would be treated with DMARDs before erosive disease is present (27). Methotrexate, Cyclosporine, Sulfasalazine and Leflunomide are the most commonly used DMARDs for severe psoriasis and PsA in clinical practice (70). It has been reported that a higher rate of toxicity has been associated with methotrexate use in PsA versus RA, resulting in earlier discontinuation of therapy (71). It is thought that methotrexate therapy may induce more hepatotoxicity in psoriasis and PsA than in RA, although the reason for this is unclear (72). Despite this, methotrexate continues to be the DMARD of choice in the treatment of PsA as a result of its effects on both arthritis and skin disease (73). Other

studies however, have indicated that Cyclosporine is also not tolerated well and likewise leads to hepatotoxicity (74).

When DMARDs fail to provide sufficient resolution, the next step is the use of biologic therapies. As will be described in greater detail in Section 1.5, TNF-α is a critical cytokine in the pathogenesis of PsA and thus is an obvious therapeutic target. TNF-α antagonists such as etanercept (synthetic TNF-α receptor), infliximab (chimeric anti-TNF-α monoclonal antibody), adalimumab (humanized anti-TNF-α monoclonal antibody), and golimumab (human anti-TNF-α monoclonal antibody) have all shown significant improvement in PsA patients according to the American College of Rheumatology (ACR) 20 and 50 criteria (73). However, it is important to realize that most reports of TNF-α antagonist therapy in PsA have focused solely on the treatment of peripheral arthritis while not assessing the effect on axial disease, dactylitis, and enthesitis. Although not specifically assessed, one trial did report that back pain and stiffness associated with PsA responded to infliximab therapy (75). Interestingly, other recent work has also suggested that infliximab may have a direct effect on angiogenesis in PsA. One study suggests that infliximab was associated with histologic evidence of decreased vascularisation in synovial tissue biopsies (76). Further, there appeared to be changes in tissue markers suggesting a reduction in angiogenesis, including reduction in vascular endothelial growth factor (VEGF). An additional study also noted similar findings in that decreased neovascularisation was seen in skin and synovial tissue after treatment with infliximab (77). Other methods of biological therapy include preventing the

activation of T cells, thus disrupting the psoriatic cascade. Medications such as Alefacept, and Abatacept block interactions between antigen-presenting cells and T cells, thus interfering with activation of T lymphocytes and migration of cells to the site of inflammation (78).

Newer targets are also being pursued as further advances in the understanding of disease pathogenesis are revealed. Ustekinumab, an inhibitor of IL-12/23 has shown efficacy in randomized control trials in psoriasis and PsA (79)(80), while other agents aimed at reducing bone erosions by inhibiting osteoclastogenesis are also being investigated for their efficacy in the treatment of PsA (81). As newer treatment options in psoriasis and PsA are approved and introduced into clinical practice, the mechanisms of the disease are further elucidated, enabling a greater understanding of the basis of pathogenesis in PsA.

## 1.6 - Pathogenesis of Psoriatic Arthritis

As with most complex diseases, historically there has been some debate as to the exact sequence of events in the development of PsA. This is further complicated by the close relationship of PsA to uncomplicated psoriasis. In recent years the immunopathogenesis has been greatly debated and reviewed extensively (82-85).

In the mid 1980s, it was discovered that T cells play a role in the pathogenesis of psoriasis; the immunosuppressive drug, cyclosporine, cleared

psoriasis. Cyclosporine was shown that to have direct antiproliferative effects on keratinocytes at therapeutic concentrations, and acts primarily through a blockade of the T cells (86). Similar improvements were seen in psoriatic patients treated with methotrexate for other disease processes. Further evidence supporting the role of T cells in psoriasis was found in the severe combined immunodeficiency disease mouse model, where the transplantation of normal appearing skin of psoriatic patients, followed by the intradermal injection of activated peripheral blood T cells of the same patient, clinically and histologically induced the appearance of psoriatic plaques (87). Likewise, psoriasis has been triggered or cured by bone marrow transplantation, depending upon whether the donor or the host had psoriasis (88,89).

### 1.6.1 - Synovial Histopathology

Skin and synovial biopsies in PsA patients have revealed that T lymphocytes are the most common inflammatory cells in the skin and joints (31,90). CD4+ T cells are the most significant lymphocytes in the tissues, with a CD4+/ CD8+ ratio of 2:1; in contrast, this ratio is reversed in the synovial fluid compartment and at the enthesis, where CD8+ T cells are more common (91,92). A dominant CD8+ T cell population in PsA synovial fluid suggests that these cells may be driving the immune response in the joint (93).This is strongly supported by the long established association of PsA with major histocompatibility complex (MHC) class I genes (46). T cell receptor (TCR) repertoire oligoclonality more commonly expressed in the CD8+ T cell population has been identified in

epidermal cells (94) and in the synovium (95) of patients with PsA. Helper T cells have classically been described as two different classes of cells: type 1 (Th1) and type 2 (Th2). Th1 cells are thought to mediate cellular immunity and secrete the proinflammatory cytokines IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α). Th2 cells are thought to mediate humoral immunity and secrete anti-inflammatory cytokines IL-4, IL-5, IL-6, IL-10, and IL-13. Studies have shown that patients with psoriasis have an increased expression of IFN-γ compared with IL-4 in circulating T cells when compared with healthy volunteers In addition, similar studies have found an increased expression of IFN-γ over IL-4 in T cells located in the epidermal layer. Thus a substantial and growing body of evidence has been generated supporting the concept that psoriasis and PsA are mediated by activated antigen-specific T cells (96), with a Th1-dominated cytokine profile (97).

PsA synovial membranes share more common characteristics with other forms of spondyloarthritis than with RA (98). Synovial biopsies from patients with PsA and other spondyloarthropathies have shown significantly less lining layer thickness and greater vascularity than those obtained from RA patients (99), and increased vascularity at both the macroscopic and microscopic level in PsA membranes have been observed (100,101). Lining layer hyperplasia is less marked in PsA and fewer macrophages are observed trafficking into the synovium and out to the lining layer, although the number of T lymphocytes and their subsets are similar in frequency to that found in RA (90). Higher neutrophil numbers are also observed, which is consistent with the well-described neutrophil infiltration seen in psoriatic skin (102). When PsA subtypes are compared,

however, no significant differences were seen between oligo- and polyarticular PsA (99).

1.6.2 - Enthesitis

Enthesis are the point at which a tendon, ligament or muscle inserts into bone, where the collagen fibres are mineralized and integrated into bone tissue. Enthesitis is a common feature in all spondyloarthropathies, and McGonagle, *et al* have hypothesized that enthesitis is a primary event in PsA with secondary spread of inflammation to the synovium (103). Imaging studies suggest that the primary site of inflammation is the entheseal insertion of joint capsule and extensor tendon, but longitudinal studies are lacking (104). Jevtic *et al.*, first described the extensive extracapsular inflammation seen on MRI scans in PsA (105). Half their patients showed extrasynovial inflammation including thickened collateral ligaments and periarticular soft tissue, particularly in dactylitic joints. In one joint, predominant extracapsular inflammation was seen without significant associated synovitis, thus raising the possibility of nonsynovial inflammation in PsA (105). However, not all patients showed evidence of extracapsular inflammation. Thus, in PsA some patients may have a predominantly synovial disease as in RA, and some may show a predominantly entheseal disease (as in undifferentiated spondyloarthropathy (SpA)). An insertion site *per se* does not appear to be a prerequisite for disease expression, for functional entheses are sites of repeated shear and compressive stressing (106).

While synovial inflammation is a cardinal manifestation of PsA, a link with enthesitis was not firmly established until recently (107). The recent recognition of a synovio-entheseal complex (SEC) may help resolve the relationship between synovitis and enthesitis in PsA (32). The SEC model shows how enthesis fibrocartilage could derive lubrication and nourishment from adjacent synovium in a manner identical to the long appreciated interaction between articular cartilage and synovium. Hence, it appears that all sites of disease in PsA can be linked to the common denominator of enthesitis.

### 1.6.3 - Bone Remodelling

Another of the hallmarks of PsA is an unpredictable pattern of bone resorption and concomitant new bone formation. The constantly changing nature of the human skeleton is not limited to perpetual bone turnover but can also be observed in the interactions between bone and other organ systems. One particularly intriguing interaction which has gained much attention in recent years is the link between the skeletal and immunological systems. The recent understanding of an interplay between adaptive immune cells and cells involved in skeletal remodelling led to the development of a field known as osteoimmunology (108,109). This rapidly expanding field has the potential to facilitate the translation of basic science knowledge in bone biology into an improved understanding of the pathological mechanisms involved in altered remodelling in inflammatory arthritis (110).

Radiographs of PsA joints reveal significantly altered bone remodelling in the form of tuft resorption, large eccentric erosions, and pencil-in-cup deformities along with features of new bone formation such as periostitis and bone ankylosis (111). With respect to bone resorption, biopsies of joints in PsA patients demonstrate large multinucleated osteoclasts in deep resorption pits at the bone-pannus junction (112). Data has indicated that osteoclast formation begins in the earliest stages of arthritis (113)

Until recently, the molecular events that underlie osteoclastogenesis were not well understood. Elucidation of the signalling pathway of the receptor activator of nuclear factor KB ligand (RANKL) and receptor activator of nuclear factor KB (RANK) has clarified some of the crucial first steps required for the process of osteoclast formation and activation (114). To summarize the process briefly, Boyle et al observed that RANKL, expressed on the surface of osteoblasts, stromal cells in the bone marrow, and infiltrating T lymphocytes and synoviocytes in the inflamed joint, binds to RANK, a cell-associated TNF receptor-related protein. RANK is expressed on a variety of cell types, including osteoclast precursors and osteoclasts. In normal bone, ongoing bone resorption mediated by osteoclasts is closely linked to new bone formation by osteoblasts (114). High expression of RANKL by synovial fibroblastoid cells coupled with diminished expression of osteoprotegerin (OPG), an antagonist of RANKL, has been observed by immunohistochemistry in psoriatic synovium (112), and an elevated RANKL/OPG ratio favours the differentiation of osteoclasts from monocytes and tips the balance towards bone resorption (107). The interaction

between RANKL and its receptor RANK in the presence of macrophage colony stimulating factor is necessary and sufficient for osteoclastogenesis and subsequent bone resorption. In addition, osteoprotegerin (OPG), a decoy receptor, can bind to RANKL and neutralize bioactivity. OPG inhibits osteolysis, and the ratio of RANKL to osteoprotegerin in a particular tissue is the primary factor determining the extent of bone resorption. Interestingly, RANKL is also expressed by T Cells which infiltrate into the synovial lining of inflamed joints (115). Further to this, blocking RANKL has resulted in some protection from cartilage destruction in arthritic animal models (116).

In addition to this, circulating osteoclast precursor cells (OPCs) were elevated in PsA patients when compared to healthy controls. Treatment with TNF-α inhibiting drugs significantly decreased the level of OCPs in peripheral blood thus providing evidence of a central role for TNF-α in the mechanism of bone resorption (112). TNF-α stimulates osteoclastogenesis via its interaction with the p55 subunit of the TNF-α receptor (TNFp55r) (117). Once bound to this receptor, TNF-α exerts several effects that foster increased osteoclast formation. TNF stimulates RANKL expression in bone marrow stromal cells and also activates the p38 mitogen-activated protein kinase (MAPK) cell-signalling pathway which leads to increased expression of colony stimulating factor 1 receptor (c-Fms). Binding of macrophage colony stimulating factor (M-CSF) to c-Fms stimulates RANK expression in osteoclast precursors. The RANKL upregulated by TNF-α in the bone marrow stromal cells binds to RANK on the

osteoclast precursors and drives increased cell signalling downstream of RANK (110).

The importance of TNF-α in this process has also been shown in animal models of inflammatory erosive arthritis. The TNF-transgenic mouse, for example, closely mimics human disease and represents the first predictive animal model of arthritis as these animals develop erosive arthritis with focal subchondral and joint margin bone erosions (118). On a cellular level, an effect of TNF-α in these animals is a four to seven-fold increase in osteoclast precursors while treatment of the TNF transgenic mice with anti-TNF-α agents restored the number of cells in this population to levels seen in their wild type littermates (119). Beyond animal models, one small study of 24 PsA patients and 12 controls examined the relationship between elevated TNF and bone-resorbing osteoclasts in PsA. The authors observed significantly increased numbers of circulating, unstimulated OCPs derived from unstimulated cultured monocytes (i.e. no RANKL or M-CSF added to the cultures) in the PsA subjects relative to controls (112). Another recent study also demonstrated that OCP frequency dropped dramatically following therapy with etanercept in patients with erosive PsA. (120).

While the mechanisms of bone resorption have recently been elucidated, the events responsible for new bone formation are poorly understood (121). Only a few factors that participate in osteoproliferation and new bone formation have been identified. Bone morphogenetic proteins (BMP) are pivotal molecules in bony ankylosis as shown in the DBA/1 mouse model (122). Aging DBA/1 male mice, when caged together, become extremely aggressive and develop an

ankylosing enthesitis, which has features that are similar to the pathology observed in psoriatic joints (123). While no studies have yet been performed on sites of new bone formation in psoriatic joints, transforming growth factor β (TGF-β) is highly expressed in synovial tissues of AS patients (124), and animal models indicate that TGF-β interacts with vascular endothelial growth factor (VEGF) to induce bone formation (125). Osteophytes, or the overgrowth of bone tissue more commonly called 'bone spurs' have been shown to express VEGF (126). The subchondral vascular system has a pivotal role in the regulation of ossification. Marginal osteophytes form when capillaries penetrate the subchondral bone and deep zone of articular cartilage. VEGF is thus likely to be an important regulator of angiogenesis during osteophyte development and thus new bone formation. (126).

1.6.4 - Angiogenesis

Angiogenesis appears to be a first-order event in both psoriasis and PsA (127). Abnormalities in the vascular morphology of the nail-folds of psoriasis patients without nail disease have been observed (128), as well as an increase in the number of synovial membrane blood vessels in PsA joint tissue (90), and as described in section 1.5.4, also plays a role in new bone formation. When viewed through an arthroscope, the blood vessels in psoriatic arthritis synovia are tortuous and dilated, with little or no branching whereas those in rheumatoid arthritis membranes have a straight pattern with regular branching, reflecting a different pathogenesis and outcome (101). The existence of these elongated and

tortuous vessels in skin and joint suggest dysregulated angiogenesis resulting in immature vessels. Angiogenic growth factors such as TGF-β and VEGF are markedly increased in psoriasis, both circulating and within psoriatic plaques (129), and VEGF and TGF-β levels have been observed to be significantly higher in the joint fluid in early PsA (130). Further, expression of angiopoietins, co-localise with VEGF protein and mRNA in PsA synovial membranes in a perivascular distribution (131).

Angiogenic targets are also being investigated for their therapeutic value: recently, a novel application of the peroxisome proliferator activated receptor-gamma (PPARG) agonist drug, pioglitazone, originally developed for the treatment of diabetes, was reported in the treatment of PsA (132). Activation of PPARG by pioglitazone has been shown to significantly reduce inflammatory cytokine expression (133) and suppress neoangiogenesis (134) in models of inflammatory disease. In this small trial, 60% of enrolled patients saw significant improvement from baseline. The benefits of this therapy are further underscored by evidence that PPARG plays a role in regulating VEGF expression (135) and that polymorphisms in the *PPARG* gene influence serum OPG levels (136,137). Thus, the evidence for angiogenesis as a common pathogenic pathway in psoriatic skin and joints is quite compelling.

## 1.7 – Heritability of Psoriatic Arthritis

Both psoriasis and PsA are highly heritable. One of the most widely accepted (and often first attempted) ways to establish the genetic proportion of a disease is to examine the concordance rates in monozygotic twins compared to dizygotic twins (138). Psoriasis is highly heritable as evidenced by an increased rate of concordance – roughly three times higher - in monozygotic versus dizygotic twins (35-72% vs. 12- 23% respectively) (139). However, concordance is never 100%, and can be as low as 35% which does suggest that environmental factors must also play an important role (140). Until early 2008, there were no published reports of twin studies in PsA, likely due to its low prevalence. The only published article resembling a twin study came from Moll and Wright (141), who reported a triplet consisting of an identical twin and a non-identical third triplet. The identical twins both developed psoriasis with one having spondylitis and the other polyarthritis. The non-identical triplet had no psoriasis or arthritis. In January of 2008, Pederson *et al.* (142) published the first true twin study in Psoriatic Arthritis, using the Danish twin registry. Following responses to a survey, 36 pairs of twins were identified with at least one twin positively diagnosed with PsA according to both the Moll and Wright (143) and CASPAR (25) criteria. According to the Moll & Wright criteria, the proband-wise concordance rates were 1/10 (10% (95% CI: 2%, 40%)) and 1/26 (3.8% (95% CI: 0.7%, 19%)) in monozygotic and dizygotic twins respectively (difference 6.3% (95% CI: -11%, 37%), p = 0.49). If the CASPAR criteria was used, the proband-

wise concordance rates were 1/9 (11% (95%CI: 2%, 44%) and 1/22 (5% (95% CI: 1%, 22%) in monozygotic and dizygotic twins respectively (difference 6.6% (95%CI: -13%, 39), p = 0.52). With regard to Ps skin disease in the PsA population (defined by the Moll &Wright criteria), the proband-wise concordance rates were 6/11 (55%) in monozygotic twins and 6/28 (21%) in dizygotic twins. Thus, based on concordance estimates, they found a significant genetic effect on Ps skin disease in PsA twins, and a substantial genetic component for the development of PsA. While the data from Pederson *et al.'s* twin study do implicate a genetic component for PsA, the numbers used to calculate remain small, again likely due to the disease's low prevalence.

Following the examination of twin concordances, the magnitude of a genetic contribution to a disease is calculated by determining the proportion of disease in the siblings (or other degree of relatives) as compared to the prevalence of the disease in the general population. This parameter – known as the recurrence risk ratio - was originally formulated by Neil Risch, and is denoted as $\lambda_R$, where R represents the degree of relatedness (144) (i.e., $\lambda_1$ would indicate a first degree relative – a full sibling, parent, or child, while $\lambda_s$ would refer to siblings only). Briefly, to calculate the $\lambda$ value for the degree of relatedness being studied, the frequency of disease in the (for example, first-degree) relatives is divided by the population frequency of the disease. The recurrence risk ratio for psoriasis is estimated to be between 4 and 10 (145).

Re-analysis of published data of psoriasis from the Faeroe Islands (15), Sweden (146) and Germany (147) revealed a $\lambda_1$ of 8 for the Faeroe Islands

cohort, 4 for the Swedish cohort, and 10 for the German cohort, indicating substantive familial aggregating of Psoriasis (140) (148). Risch also developed a formula for using risk ratios among relatives of different degrees to obtain information about genetic models. When the risk ratio ($\lambda_{R\ -1}$) decreases by a factor of greater than 2 between the first and second degrees of relatedness, the data are consistent with a multilocus model. As this factor was 7 in the Faeroe Islands study and 8 in the Swedish study, a multilocus model for psoriasis is predicted.

Due again to the low prevalence of PsA, the number of studies estimating the familial aggregation of PsA are quite low in number. Moll and Wright's (141) study in 1973 assessed first and second degree relatives of 88 probands with PsA. There were a total of 181 first-degree relatives, of which 10 had PsA, including 5 siblings indicating that the overall prevalence of PsA among first degree relatives is 5.5%. The prevalence of PsA in the UK at the time was accepted as being approximately 0.1%, therefore the $\lambda_1$ risk for first degree relatives of PsA probands, was 55 – a number significantly higher than that for relatives of psoriasis probands, as described above.

More recently, two reports have published new data on the familial recurrence risk of PsA. Meyers *et al.* (149) examined 80 PsA probands and 112 siblings who presented at the Rheumatology department of Freeman Hospital at Newcastle-Upon-Tyne, UK. Sibling prevalence was reported at 14.3% making the $\lambda_s$ 47. Chandran *et al.* (150) examined 100 consecutive probands who had 396 total first degree relatives. 289 of these participated (73%) (130 sibs, 108 parents,

51 children). The authors assumed a population prevalence of 0.25% for PsA and 2% for Ps as described previously by Gelfand *et al.* (18) It was found that the prevalence of PsA was 7.6%, and the prevalence of psoriasis was 15.2% among first degree relatives. Therefore, $\lambda_1$ was 30.4 for PsA and 7.6 for psoriasis. With respect to the siblings, the $\lambda_s$ was found to be 30.8 for PsA and 8.8 for psoriasis. To put these numbers into perspective, the $\lambda_s$ for RA is estimated to be between 2 and 17 (151), and for Type 1 Diabetes is 15 (152).

Interestingly, an epigenetic mode of inheritance has been observed in PsA. Rahman *et al.* (153) observed excessive paternal transmission (i.e., genetic imprinting) when it was discovered that the proportion of probands with an affected father (0.65) was significantly greater than the expected proportion of 0.5 ($p = 0.001$). Similar trends were noted in the offspring and second degree relatives of the proband. Imprinting, exemplified by the Prader-Willi/Angelman syndromes, has been implicated in other auto-immune diseases such as insulin-dependent diabetes mellitus (IDDM) and inflammatory bowel disease (IBD). This paternal transmission has also been reported in Icelandic PsA families (154). Taken cumulatively, these family data argue for a substantial genetic component to both psoriasis and PsA.

## 1.8 - Review of the Genetics of Psoriatic Arthritis

### 1.8.1 – Genetic Basis of Psoriasis

Psoriasis and PsA are interrelated disorders, as most PsA patients have psoriasis (1). Psoriasis is known to be an immunologically mediated disease, however the molecular events triggering this immune-activation remain unclear. Psoriasis has long been accepted as having a strong family history, and as described in section 1.6, Chandran *et al* recently demonstrated that psoriasis is highly heritable with a $\lambda_1$ of 7.6 and a $\lambda_s$ of 8.8 (150).

Several regions of the genome have been linked to susceptibility for the development of psoriasis, with the most consistently observed region being a locus within the Major Histocompatibility Complex (MHC) region at chromosome 6p21.3 (155,156). This locus has been named *PSORS1* (**Pso**riasis **S**usceptibility region **1**) and association studies have consistently demonstrated an association with the *HLA-Cw6* allele across several different populations (157,158). It is estimated that this region accounts for one-third to one-half of the genetic susceptibility to psoriasis (159).

Recent analysis has confirmed that *HLA-Cw6* is the variant in the MHC region which predisposes to psoriasis (160). Psoriasis affects males and females in equal proportions and can occur at any age (161). Carriage of the *HLA-Cw6* allele is most common in those with an early age of onset and family history (i.e., Type I Psoriasis) (162) (it is worth noting, however, that despite the strong association with *HLA-Cw6*, not all psoriasis patients carry the allele. In

independent sets of affected individuals/families it is only found in 40–80% of cases. Moreover the penetrance of *HLA-Cw6* is 10%, implicating environmental effects or additional genetic susceptibility factors (163)). While the *PSORS1* locus is generally understood to confer the most significant risk for the development of psoriasis, several other susceptibility loci also have been identified outside of the MHC region. Linkage scans have implicated other loci designated as *PSORS2-PSORS10;* however in many of these replication has been difficult to achieve and statistical significance is often low (164). Of these, the *PSORS2* (17q24-25), *PSORS5* (3q21) and *PSORS9* (4q28-31) loci show the strongest evidence for replication. Four independent linkage studies have shown significance ($p < 0.01$) for the *PSORS2* region following the original report, while another reported significance at $p = 0.05$ (155,165-169). *PSORS5* has likewise been replicated (170,171), while a meta analysis of four different genome wide linkage scans using Caucasian and Chinese Han populations observed significance in only the *PSORS1* and *PSORS9* loci (172). A summary of all implicated *PSORS* regions is included in Table 1-4.

As will be described in more detail in section 1.7.4, the few genome-wide association studies performed in psoriasis have found significance for SNPs in the MHC region, as well as variants of the *Il-23R* and *Il-12B* genes as well as two genes that act downstream of TNF-α and regulate NF-κB signalling (*TNIP1, TNFAIP3*).

## Table 1-4: Summary of Known Psoriasis Susceptibility Regions

| Locus | Region | Gene Candidates | Lead Author and Year of Publication of Psoriasis Susceptibility Studies |
|---|---|---|---|
| *PSORS1* | 6p21.3 | *HLA-Cw6; CDSN, HCR, HERV-K, HCG2, 7PS04S1C3, POU5F1, TCF19, CCHCR1, LMP, SEEK1, SPR1* | Samuelsson L, 1999; Lee YA, 2000; Elder JT, 2001; Veal CD, 2001; Zhang XJ, 2002; Foerster J, 2004; Sagoo GS, 2004 |
| *PSORS2* | 17q25 | *RUNX1; RAPTOR; SLC9A3R1; NAT9; TBCD* | Tomfohrde J, 1994; Nair RP, 1997; Enlund F, 1999; Samuelsson L, 1999; Helms C, 2003; ZhengY, 2003; Capon F, 2004; Stuart P, 2006; Capon F, 2007 |
| *PSORS3* | 4q34 | *IRF-2* | Matthews D, 1996; Hida S, 2000; Foerster J, 2004 |
| *PSORS4* | 1q21 | *Loricrin; Filaggrin; Pglyrp3,4; S100 genes within epidermal differentiation complex* | Bhalerao J, 1998; Capon F, 1999; Semprini S, 2002; Giardina E, 2004; Giardina E, 2006; Sun C, 2006; Zhao Y, 2007 |
| *PSORS5* | 3q | *SLC12A8; Cystatin A; Zn finger protein 148* | Enlund F, 1999; Samuelsson L, 1999; Hewett, 2002; Samuelsson L, 2004; Huffmeier U, 2005 |
| *PSORS6* | 19p13 | *JunB* | Lee YA, 2000; Zenz R, 2005 |
| *PSORS7* | 1p | *PTPN22 (1p13); IL-23R (1p32.1–31.2)* | Veal CD, 2001; Tsunemi Y, 2002; Nistor I, 2005; Duerr RH, 2006; Huffmeier U, 2006; Capon F, 2007;Cargill M, 2007 |
| *PSORS8* | 16q | *CX3CL1, CX3R1; NOD2/CARD15* | Nair RP, 1997; Karason A, 2003; Young C, 2003; Plant D, 2006 |
| *PSORS9* | 4q31 | *Il-15* | Bhalerao J, 1998; Samuelsson L, 1999; Zhang XJ, 2002; Bowcock AM, 2004; Sagoo GS, 2004; Sun LD, 2007; Zhang XJ, 2007 |
| *PSORS10* | 18p11 | - | Veal CD, 2001; Asumalahti K, 2003 |
| - | 5q31.1–33.1 | *IL-12B; SLC22A4; SLC22A5; IL-13; IL3, IL4, IL5, CSF2 and IRF1* | Tsunemi Y, 2002; Duerr RH, 2006; Friberg C, 2006; Capon F, 2007; Cargill M, 2007, Nair et al, 2008 |
| - | 9q33-34 | - | Zhang XJ, 2002; Yan KL, 2007 |
| - | 6p22 | *CDKAL1* | Wolf N, 2007 |
| - | 19q34 | *KIR2DS1, KIR2DL1, KIR2DL5* | Suzuki Y, 2004; Luszczek W, 2004 |

*Adapted from* (176)

1.8.2 - Genes in the MHC Region Associated with PsA

The MHC locus spans approximately 4 Mb and contains roughly 250 genes, of which approximately 60% have immune-related functions. The MHC region is characterized by extended LD blocks (up to 3 Mb), and by a strong and complicated LD pattern between the blocks (173). Nair *et al.*'s work in 2006 demonstrated conclusively that the *PSORS1* gene is in fact the *HLA-Cw*0602* allele (160). While the strong association of *HLA-Cw*0602* also exists for PsA, it is predominantly associated with a younger age at onset of psoriasis (< 40 years, i.e., Type I Psoriasis) in PsA patients, suggesting that the association is primarily with psoriasis rather than PsA *per se* (174,175).

In addition to *HLA-Cw6*, both *HLA-B7* and *-B27* are associated with PsA associated with later onset of arthritis in PsA patients (46). (Interestingly, *HLA-B27* is also the strongest reported association with Ankylosing Spondylitis, another seronegative spondyloarthropathy.) Further to these, other HLA markers identify increased or decreased risk for disease severity and progression: *HLA-B39* alone, *HLA-B27* in the presence of *HLA-DR7*, and *HLA-DQw3* in the absence of *HLA-DR7* predispose for additional risk for disease progression; independent of psoriasis (46,177). Specifically, *HLA-B27* is associated with the spondyloarthritis (spinal involvement) form of PsA, and as well with distal interphalangeal (DIP) joint involvement (178). Conversely, the presence of *HLA-B22* protects against disease progression (179). The RA 'shared epitope' (*HLA-DRB1*01* and *HLA-DRB1*04*) and severity of PsA are associated: patients who possessed at least one copy of the shared epitope had significantly more erosive

disease than those who did not (180). Recently it was also found that carrying both *HLA-Cw6* and *HLA-DRB1*07* alleles predisposed PsA patients towards a less severe course of arthritis (181).

Associations have been reported with two other non-HLA genes in the MHC region and PsA, both of which have been replicated. As evidenced through its involvement in multiple facets of the psoriatic and inflammatory pathways described earlier, variations in the *TNF-α* gene which is located 250kb centromeric from the *HLA-B* locus are likely to play a role in PsA. Several studies examining promoter polymorphisms in *TNF-α* with conflicting results have been published (174,182,183). The most consistent results have been with the promoter SNPs at positions -238 and -308 relative to the start of transcription. The -238 and -308 SNPs have shown association across several Caucasian populations (174,182,184). Interestingly, other work has shown the -308 polymorphism to be associated both with more erosive change in PsA patients (185), and with serum levels of circulating TNF-α. A recent study has reported that the -857 SNP may be a risk factor for PsA but not for psoriasis that is independent of the *PSORS1* locus (186).

The other replicated non-HLA MHC gene is the major histocompatibility complex class I-related gene A (*MICA*). The *MICA* gene is a highly polymorphic gene situated in the human leukocyte antigen (HLA) class I region, located 47 kb upstream from the HLA-B locus, and extensive linkage disequilibrium (LD) between HLA-B and *MICA* has been described (187-189). A triplet microsatellite repeat in the transmembrane region of exon 5 of the *MICA* gene was found to be

associated with a number of immunologically mediated disorders such as RA (190), Bechet's Disease (191), and AS (192-194). The location of the *MICA* gene and its extensive LD with HLA alleles associated with PsA, as well as its association with the previously described diseases indicate that it is an interesting candidate gene for PsA, and several studies have demonstrated an association between *MICA* and PsA across several populations (183,195,196). The reported association was found to be independent of *HLA-Cw*0602* and psoriasis in a Spanish population (197), and was also replicated in an Israeli PsA cohort (198). *MICA* represents one of the few genetic associations that appears to be strictly involved with PsA and not psoriasis, as no evidence of an association with psoriasis has yet been published.

Two other non-HLA MHC genes have been identified as potential candidate genes in PsA however the evidence is less well established. The gene known as *PsorS1C1* (also referred to as *SEEK1*) has been suggested as a candidate gene, however, its theorized involvement is due entirely to location as it overlaps the 6p21-23 region and is slightly telomeric to and in LD with the *HLA-C* locus, was noted to have a strong association with psoriasis in the Swedish population and its effect was independent of *HLA-Cw*0602* (199). However, the function of this gene remains unknown. The Corneodesmosin (*CDSN*) gene is found roughly 160kb telomeric from *HLA-C*, and several reports have described associations with psoriasis (as well as LD with *HLA*-C) (200-202). *CDSN* makes an interesting candidate gene in psoriasis and PsA as it is the only transcript from the *PSORS1* region to be expressed in well differentiated keratinocytes (163),

and as well polymorphisms of this gene are known to increase mRNA stability, leading to a potential pathogenic mechanism (203).

1.8.3 - Non MHC Genes

While there are many candidate genes in PsA based on either location or putative function in a biological pathway, there is a paucity of replicated candidate gene studies. The IL-1 gene cluster on chromosome 2q12-13 is an area of special interest as the expression of IL-1 is known to be upregulated in both the serum and synovium of patients with PsA (204). Ravindran *et al* (205) noted an association with the IL-1 alpha 889C polymorphism, however, while this specific SNP was not replicated (206), an association with PsA was noted with a haplotype of other SNPs in the extend IL-1 family gene cluster (207).

The Killer Immunoglobulin-like Receptor (*KIR*) genes encode receptors on Natural Killer (NK) cells. The *KIR* gene cluster is highly polymorphic, nearing the level of variation observed in the MHC (208). The *KIR* receptors interact with MHC class I molecules, in particular those encoded by several *HLA-C* alleles (209). Once this interaction occurs, the threshold for triggering a T-cell by engagement of the classical T-cell receptor with an antigen presented by MHC molecules is lowered, while the presence of an inhibitory receptor will raise the threshold necessary for T-cell activation. An association has been observed between polymorphisms in the genes *KIR2DS1* and *KIR2DS2* with both PsA (210,211) and psoriasis (212), but only if the HLA ligand for the corresponding inhibitory receptors (KIR2DL1 and KIR2DL2/3) were absent. Martin *et al* (213)

have proposed a model of how certain HLA and KIR genotypes can influence susceptibility to PsA, which postulates the mechanism for a pathogenic role for HLA-Cw*0602 in the development of psoriasis and PsA.

A recent genome wide association study noted an association between psoriasis and a haplotype in the *Il-23R* gene (214). This was quickly replicated (215,216). Interestingly, one of the SNPs in this haplotype is also associated with Crohn Disease as a protective allele (217). As there is evidence that IL-23 is involved in regulating Th1 pathway responses (218) and it has been detected in psoriatic skin (219), the *Il-23R* gene made an obvious target for investigation in PsA. Two studies have replicated an association of *Il-23R* with PsA (220,221). It is interesting to note, however that a recent study reports that synovial fluid, serum and synovial biopsies from 30 non-PsA SpA, 22 PsA and 22 RA patients do not show an increase in levels of IL-23R for SpA or PsA patients. An increase was observed in RA patients, suggesting that IL-23 is likely to be involved in psoriasis rather than PsA (222).

The protein tyrosine phospatase non-receptor type 22 (*PTPN22*) gene is expressed only in hematopoietic cells, acts as a regulator of the negative regulatory kinase cytoplasmic tyrosine kinase (CSK) in T cells, and may play a role in suppression of T-cell activation (223). A functional SNP at nucleotide position 1858 C>T causing an Arg→Trp substitution (R620W) which disrupts the binding site for CSK is associated with IDDM (224), and other autoimmune diseases including RA (225) and systemic lupus erythematosus (SLE) (226) in Caucasian populations. To further examine the role of *PTPN22* a collection of

265 multiplex families was assembled by the Multiple Autoimmune Disease Genetics Consortium (MADGC) (227). At least two of nine "core" autoimmune diseases (RA, SLE, IDDM, multiple sclerosis (MS), autoimmune thyroid disease (Hashimoto thyroiditis or Grave's disease), juvenile RA, inflammatory bowel disease (Crohn disease or ulcerative colitis), psoriasis, and primary Sjogren syndrome) were present in each of these families. They observed that the *PTPN22* 1858 C>T polymorphism confers risk of four separate autoimmune phenotypes in these families: T1D, RA, SLE, and Hashimoto thyroiditis, suggesting a common underlying etiologic pathway for some, but not all, autoimmune disorders. No association was observed with psoriasis in the MADGC study, however, only 63 psoriasis patients were part of the study group. Since that time, several other studies have been conducted examining the association of *PTPN22* with both psoriasis and PsA, and significant associations with both have been observed (228-231). The clearly evident association of *PTPN22* with multiple autoimmune diseases makes it a strong candidate for continued investigation in PsA.

### 1.8.4 - Genome Wide Scans

Only one traditional genome wide linkage study has been performed in PsA, using a large Icelandic cohort. An initial LOD score of 2.17 on chromosome 16q21 was found, however this was increased to 4.19 when conditioned on paternal transmission (154). A subsequent study and the use of additional markers further raised the LOD score to 5.7 (232). The peak of this LOD score

was within 20Mb of the gene *CARD15* which has been associated with Crohn Disease (233). One study found an association between this gene and PsA (234). While this finding has not been replicated (235-237), a recent study has shown evidence of an interaction between *HLA-Cw6* and *CARD15* in psoriasis families (238).

Likewise, only one genome wide association study has been performed using a distinct PsA cohort (239). The study, which used both psoriasis and PsA cohorts from the US and the UK appears to have confirmed the associations with *IL-12/23R* and PsA. It is worth noting, however, that more recent reports suggest that these variants are not specific risk factors for arthritis, but relevant for susceptibility to psoriasis in general (240). A recent psoriasis genome wide association study that included two cohorts with high levels of PsA would appear to add further evidence to this contention (241).

The results of the PsA genome wide association study also confirmed that the MHC is truly the most important risk factor for psoriasis and that it plays a very major role in PSA, as the most highly associated SNP from the study was found 34.7 kb upstream from *HLA-C* (U.K. PSA: $p= 6.9 \times 10^{-11}$). The highest odds ratio was found for a coding SNP in the MHC class 1 gene *HCP5* (OR PsA 3.2, Psoriasis 4.1) and the effect was independent of the upstream *HLA-C* SNP. A novel PsA (and potentially psoriasis) locus was also identified on chromosome 4q27 (239). This region contains the *Il-2* and *Il-21* genes and was recently shown to be associated with autoimmune diseases such Celiac disease, Type 1 diabetes, Grave's disease and Rheumatoid Arthritis (242-245).

## 1.9 - Review of the Genetics of Related Diseases

### 1.9.1 - Ankylosing Spondylitis

Between 40 and 50% of PsA patients have spinal involvement, similar to ankylosing spondylitis (AS). AS is one of the most common forms of chronic, inflammatory arthritis: it is seronegative for rheumatoid factor (RF), affecting joints in the spine and sacroilium in the pelvis, causing eventual fusion of the spine (246). It has an estimated prevalence of 0.1–0.9%, and genetic factors have been strongly implicated in its aetiology, with a heritability estimated to be >90% (247). The mode of inheritance is unclear; however, the recurrence risk ratio suggests a multilocus, multiplicative model (248). A very strong association between AS and *HLA-B27* has been established for decades (249), and it is observed in over 90% patients with AS. However, *HLA-B27* is not the sole MHC molecule implicated in AS susceptibility. The entire genetic contribution of the MHC is estimated to be 50% of which HLA-B27 accounts for 16% of the effect (250). Only approximately 8% of individuals with *HLA-B27* develop AS, and 10% of Caucasian AS patients do not carry *HLA-B27* (251).

Three genome wide linkage studies have been performed in AS patient families. These data were re-analysed and then pooled for a meta-analysis by Carter *et al* (252). Not surprisingly, the MHC region showed the highest significance for linkage ($p < 10^{-5}$) to a 37–75 cM region of chromosome 6, consistent for linkage with the location of HLA-B27. The next strongest evidence of linkage was found at chromosome 16q ($p = 1.8 \times 10^{-4}$) overlapping the region

previously identified as displaying linkage to PsA. Other regions achieving moderate evidence of linkage ($p < 0.01$) were identified on chromosomes 3, 10 and 19. Nominal evidence of linkage was found for the region containing the *IL-1* gene cluster on chromosome 2q, and with the *CYP2D6* gene on chromosome 22. Both of these genes had previously reported associations with AS (207,253-256). While no true genome wide association study has been performed on AS patients, the Wellcome Trust Case Control Consortium (WTCCC) did examine 14436 nonsynonymous SNPs and a further 897 SNPs in the MCH region, from 1000 AS cases and 1500 controls (242). This is by far the largest association study completed to date for AS, but nonetheless has only investigated a small proportion of the overall genetic diversity in relation to the disease (257). In addition to the expected associations of the SNPs from MHC region, significant associations were also identified with the genes *IL-23R* and *ERAP-1* (also known as *ARTS1*). The finding of association with *IL-23R* is particularly interesting as it seems to provide evidence that variants of the gene are involved in bone disease, which counters the previously discussed contention that despite being associated with both PsA and psoriasis, *IL-23R* was more likely to be associated with psoriasis rather than PsA. Subsequent to the WTCCC study, the association of *IL-23R* with AS has been replicated in Spanish (258), Canadian (Newfoundland) (259) and UK (260) cohorts.

1.9.2 - Crohn Disease/Inflammatory Bowel Disease

It has long been recognized that patients with Crohn disease have an increased risk for the development of PsA and psoriasis (261-263). Both acute and chronic inflammation have been reported in the bowel of 15% of PsA patients (264) and 30% of psoriasis patients (265). These bowel lesions range from asymptomatic focal microscopic inflammation to inflammatory bowel disease (IBD) with macroscopic and histologic features indistinguishable from Crohn disease (266). As described previously, the only linkage study performed in PsA identified a linkage peak within 20 Mb of the known Crohn susceptibility gene *CARD15* on chromosome 16q21. Linkage studies in Crohn/IBD indicated that another strong susceptibility region was located at chromosome 5q31 known as the *IBD 5* locus (267,268). Subsequent investigation of the organic cation transporter (*OCTN*) gene cluster in this region revealed an association of a functional haplotype of two SNPs of the SLC22A4 and the adjacent *SLC22A5* genes with Crohn disease (269). Given the known association with Crohn and PsA, Ho *et al* examined this haplotype in a PsA cohort, a psoriasis cohort, and an undifferentiated inflammatory arthritis cohort, and observed an association only with PsA (270), lending evidence that there is commonality in the genetic predisposition for inflammatory diseases such as Crohn and PsA (269).

At the time of this writing, there have been more than 30 replicated genes identified in Crohn Disease. The most common associations are the *CARD15* and *IBD5* loci, but several others found through genome wise association studies have also been replicated. These associations are summarized in table 1-5.

**Table 1-5: Summary of selected genetic associations with Crohn Disease, replicated by genome wide association studies**

| Gene/Locus | Region | Lead Author and Year of Publication (Replication) |
|---|---|---|
| *CARD15* | 16q12 | Rioux, 2007<br>Hampe, 2007 |
| *IBD5* | 5q31 | Raelson, 2007<br>Hampe, 2007 |
| *IL-23R* | 1p31 | Libioulle, 2007<br>Franke, 2007<br>WTCCC, 2007 |
| *ATG16l1* | 2q37 | Libioulle, 2007<br>Rioux, 2007<br>WTCCC, 2007 |
| *MST1* | 3p21 | Parkes, 2007<br>Raelson, 2007 |
| *PTGER4* | 5p13.1 | WTCCC, 2007<br>Parkes, 2007 |
| *IRGM* | 5q33 | Parkes, 2007 |
| *TNFSF15* | 9q32 | WTCCC, 2007 |
| *PTPN2* | 18p11 | Parkes, 2007 |

*Adapted from* (274)

Several published studies based on a candidate gene hypothesis have identified an association between nuclear-factor kappa-beta 1 (*NFKB1*) and IBD (271-273). *NFKB1* is a subunit of the NF-κB protein. NF-κB is a pleiotropic transcription factor which is present in almost all cell types and is involved in

many biological processes such as inflammation, immunity, cell differentiation, cell growth, tumourigenesis and apoptosis, and as described in section 1.5.3, it interacts directly with its receptor activator *RANK* in a central role in osteoclastogenesis (275-277). Interestingly, it has also been tentatively associated with psoriasis (278,279) and is directly involved in both the TNF-α receptor pathway (280,281) and in IL-23 mediated signalling (282,283).

A total of seven different genome wide association studies in several different populations (Asian, North American Caucasian, UK Caucasian, French Canadian) have been performed in Crohn disease, and interestingly, several have detected associations with *IL-23R*. The results have indicated both protective associations as well as disease-predisposing alleles (284). The repeated association of *IL-23R* with interrelated inflammatory immunologically mediated conditions such as PsA, psoriasis, AS, and Crohn/IBD clearly indicated that it is a multi-functional gene involved in systemic inflammation.

### 1.9.3 - Rheumatoid Arthritis

A detailed description of the genetics and pathogenesis of RA is not warranted as the disease and most implicated genes are substantially different from the disease process and genetics of PsA. The non-HLA genes which have been implicated in RA are summarized in table 1-6. A brief description of RA is included only to illustrate the difference between both diseases. For a considerable period of time, PsA was simply considered to be the co-occurrence of psoriasis and RA. RA is a chronic, systemic, inflammatory autoimmune

**Table 1-6: Summary of selected genetic associations in RA.**

| Gene/Locus | Region | Lead author and year of publication |
|---|---|---|
| MMEL1/TNFRSF14 | 1p36.32 | WTCCC, 2007 |
| PADI4 | 1p36.13 | Suzuki, 2003 |
| PTPN22 | 1p13.2 | Begovich, 2004 |
| STAT4 | 2q32.3 | Amos, 2006 |
| CTLA4 | 2q33.2 | Plenge, 2005 |
| TNFAIP3/OLIG3 | 6q23.3 | Thomson, 2007 |
| C5/TRAF1 | 9q33.2 | Plenge, 2007 |
| PRKCQ | 10p15.1 | WTCCC, 2007 |
| KIF5A/PIP4K2C | 12q13.3 | WTCCC, 2007 |
| CD40 | 20q13 | WTCCC, 2007 |

*Adapted from* (285)

disorder causing symmetrical polyarthritis of large and small joints, typically presenting between the ages of 30 and 50 years (286). Approximately 1% of the global population has RA, and it is found 3 times more frequently in females than in males. Like PsA the aetiology of RA is not fully understood but involves a complex interplay of environmental and genetic factors. The typical presentation is polyarticular, with pain, stiffness, and swelling of multiple joints in a bilateral, symmetric pattern. A minority of patients present with oligoarticular asymmetric involvement (287). As PsA is largely asymmetrical, the symmetry of the arthritis

present is one of the characteristics which helps differentiate PsA from RA, as well as the absence of the rheumatoid factor (RF) antibody, axial involvement, enthesopathy, and association with *HLA-B27* (288). While approximately 30% of RA patients will be RF seronegative (289), the previously described criteria for diagnosis of PsA (Moll & Wright, CASPAR) help distinguish these cases from RA.

Variation in the HLA region has been associated with RA since the late 1970's (290) and is the only region that has been consistently shown to be both linked and associated with RA across all populations. Whereas the association with PsA has been repeatedly shown to be with the *HLA-Cw*0602*, the HLA association with RA is with specific alleles of the class II gene, *HLA-DRB1*, that encode a conserved sequence of amino acids in the third hypervariable region (HVR3) of the class II DR-1 chain. These associated alleles are collectively referred to as the shared epitope (SE) (291). As described in section 1.7.3, the MADGC initially reported an association of RA with the *PTPN22* gene, and this finding has been replicated several times in highly powered studies of populations of European descent (151,292-294). The WTCCC study also confirmed that the two strongest associations for RA are for *HLA-DRB1* and *PTPN22*. Several recent reviews and genome wide association studies have reported extensive lists of SNPs associated with RA (285,295-298), however, of these the only overlap with reported associations with psoriasis or PsA remains the *PTPN22* gene. While there are different HLA associations with RA and PsA reflecting the different pathogenesis of both diseases, the data regarding the

reported associations of both diseases with *PTPN22* indicate that there may be common underlying pathways in inflammatory arthritis.

### 1.9.4 – Shared Genetics of Immune-related Diseases

There exists a large spectrum of immunologically mediated diseases, from the seronegative spondyloarthropathies such as PsA and AS, as well as RA, to other non-arthritic diseases such as inflammatory bowel disease, type 1 diabetes and asthma. While genetic associations have long been reported for each of these diseases, an increase in co-morbidity and clustering of different autoimmune diseases in families suggest the existence of an overlap in the genetic background of these diseases (299,300). Until recently, only the HLA locus and a few candidate genes have consistently been associated with these immunologically mediated conditions, however, with the development of genome-wide association studies, a growing number of candidate genes and loci have been and continue to be identified.

Recently, Zhernakova *et al.* reviewed 22 genome wide association studies on 11 different immune mediated disorders (300). The authors observed that the major region linked to immune-related disorders was the HLA region, and also identified 23 genes that are shared by two or more diseases. While the prevailing line of thought has maintained that autoimmune disorders develop through different mechanisms from those that cause inflammatory disorders, many of these newly identified loci are shared by two or more immune-related diseases, and the majority of these shared genes belong to just a few immunological

pathways: T-cell signalling and differentiation, innate immunity, and TNF signalling. Further, many of the disease-specific associated genes are involved in the same pathways or were members of two functional groups - one group consisted of genes that are shared between ulcerative colitis and Crohn disease (IBD), and the other group encoded cytokines and chemokines. Several of the disease-specific genes identified also belong to the three immunological pathways. Interestingly, at least one associated gene was involved in each pathway for almost all of the immune diseases that were studied, which highlights the overlap in these immunological pathways between diseases. (The overlap of genes in selected immune-related diseases is shown in Figure 1-2). Fully elucidating the shared pathogenesis between various immunologically mediated diseases has the potential to clarify new targets for therapeutic intervention by focusing on pathways rather than genes.

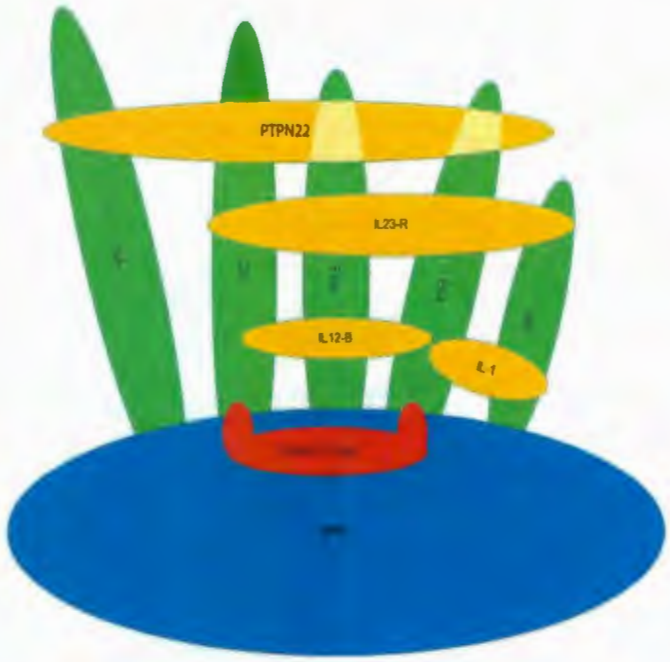


**Figure 1-2: The overlap of genes associated with multiple auto-immune diseases**
(The area of the overlap between *PTPN22* and Psor and PsA is left transparent as this association has not yet been confirmed. All other associations shown have been replicated.)

*Adapted from Zhernakova et al., 2009*
*Special thanks to Mr. Lance Doucette*
*For his assistance in composing this figure*

## 1.10 - Rationale and Goals for Study

Broadly, the goals for this thesis are to confirm existing genetic associations with PsA, to identify novel gene associations and gene-gene interactions in PsA. As described previously, a large number of studies have been performed using several different ethnic populations from different locations around the world attempting to associate variations of the genes involved in different aspects of the pathogenesis of PsA. These studies have frequently produced conflicting results, due to such features as relatively small sample sizes, a low frequency of the gene variant being investigated, and ethnic heterogeneity of the populations being investigated.

As PsA is a complex disorder, the genetic component of PsA is likely to be the result of a combined effect of a number of genes, with each playing only a small role. One of the largest and most common challenges facing case control studies is the ethnic heterogeneity of the investigated populations. Newfoundland presents an interesting and unique population structure which can help to overcome the heterogeneity frequently found in other studies.

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean, off the eastern coast of Canada. The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the late $18^{th}$ and early $19^{th}$ centuries, although settlements dating to the 1600's have been recorded (301). The settling of Newfoundland is unique when compared to colonization of other areas of the

New World, as the majority of those who immigrated to Newfoundland can be traced almost exclusively back to small regions in southwest England (the neighbouring counties of Cornwall, Devon, Somerset, Dorset, and Hampshire) and southeast Ireland (roughly a 30 mile radius from the city of Waterford). These areas were the principal ports of the United Kingdom's fishing industry at the time (302). The major migrations concluded in the mid-19$^{th}$ century at which point the population of the island was approximately 75,000 individuals. From this limited number of founding individuals, the population grew almost exclusively through expansion as the geographical and social isolation of this island has ensured very little inward migration since it was settled (303) and thus has lead to a small population (507 900 individuals; Statistics Canada 2008 – 50% of whom still live in communities smaller than 2500 individuals). While Newfoundland does not represent a true population isolate or founder population, showing levels of linkage disequilibrium comparable to that of an outbred European derived sample and the Afrikaner population of South Africa (304), there is evidence of relatively homogenous genetic background as demonstrated by a higher than expected prevalence of several Mendelian disorders (305). In addition, mating segregation between Irish Catholics and English Protestants, and geographical isolation of communities have resulted in several small population isolates, that have become more admixed as time and technology have allowed increased access into and out of the outports (303). In a review of the historical development of genetic isolation, it was observed that only 1% - 8% of breeding parents were immigrants to the area, and 60% of births had been to parents originating from the same

small community (306). As the Newfoundland population is almost entirely derived from settlers who shared a common geographic location and culture for several generations, the shared background combined with extensive genealogy from the current population are ideal for the study of complex disease such as PsA (305). The limitations of using a population such as Newfoundland in genetic studies are that the results may not be generalizable to more admixed population structures. In order to account for this, we have frequently used a PsA cohort from Toronto to validate our findings in the Newfoundland population.

# 2

# MATERIALS AND METHODS

## 2.1 – Patient and Control Ascertainment

PsA was defined as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. All patients from the Newfoundland cohort were ascertained by a qualified rheumatologist (Dr. Proton Rahman). All patients were over the age of 18 years and were voluntary participants in this research study, who presented at rheumatology clinics under the Eastern Health management board in St. John's, NL, Canada. A protocol for clinical investigation for this study was approved by the Human Investigations Committee of the Faculty of Medicine of Memorial University of Newfoundland, and by the Eastern Health healthcare management board of St. John's, NL, Canada. All patients were Caucasians of northern European ancestry. All controls were ethnically matched, taken from the Newfoundland population, as well as matched for age and gender. Control subjects were originally participants in other research studies for conditions, that had had no pathological relationship to any form of skin disorder or arthritis, and were examined to sure there were no other auto-immune condition.

All patients from the Toronto cohort were Caucasian, were also ascertained by a qualified rheumatologist (Dr. Dafna Gladman) and were over the age of 18 years and were voluntary participants in this research study. The Toronto controls either were ascertained from the local HLA laboratory DNA bank which includes healthy volunteers and organ donors, or were population based. No information regarding age or gender was available for control subjects from

the Toronto cohort, and ancestry was presumed to be Caucasian based on individual surname.

## 2.2 - DNA Extraction from Whole Blood

All DNA samples were extracted from white blood cells of venous blood which was collected from subjects in EDTA Vacutainer tubes. DNA was extracted from PsA probands and age and gender matched controls using the Promega Wizard Genomic DNA purification kit (307). Briefly, blood was added to a tube containing cell lysis solution and the components were mixed by inversion several times and allowed to incubate at room temperature for 10 minutes in order to lyse red blood cells. The mixture was then centrifuged at 2000 x g for 10 minutes. The supernatant was poured off without disturbing the remaining pellet of white blood cells, until approximately 100 µl of residual liquid remained. The pellet was then vortexed vigorously to resuspend the white blood cells, and nuclei lysis solution was added to the resuspended cells. The solution was pipetted 5-6 times to lyse the white blood cells. RNase solution was then added to the nuclear lysate and mixed by inversion. The mixture was incubated at 37°C for 15 minutes and cooled to room temperature. Protein precipitation solution was added to the nuclear lysate and the solution was vortexed vigorously for 10-20 seconds, and centrifuged at 2000 x g for 10 minutes. A brown pellet containing the precipitated

protein was then visible. The supernatant containing the DNA was transferred to a clean 15 ml centrifuge tube containing room temperature isopropanol. This mixture was gently mixed by inversion until DNA was visible, and was then centrifuged at 2000 x g until it formed a small white pellet. The supernatant was carefully decanted so as to not disturb the DNA pellet, which was then gently washed 70% ethanol by inversion, and centrifuged at 2000 x g. The ethanol was gently aspirated using a pipette and the tube was inverted on clean absorbent paper leaving the pellet to air dry for 10-15 minutes. 250µl of DNA Rehydration Solution was added to the tube and incubated for 1 hour at 65°C. Concentration of extracted DNA was determined by spectrophotometry, and was then transferred to a clean, labelled 2000µl eppendorf tube and stored at -80°C.

## 2.3 - Marker Selection

The pathogenesis of PsA has been described in Chapter 1 as being the combination of several distinct mechanisms, involving components situated in cytokine-induced inflammation, enthesitis, bone remodelling, and angiogenesis. Thus, we have opted for a comprehensive approach to examine genetic markers potentially involved in all facets of the pathogenesis of PsA. A candidate gene approach is used to investigate novel associations, while meta-analysis is used to determine the proportion of involvement for markers which have been replicated in other populations. A haplotyping approach is employed in our admixed cohort

to attempt to validate an observed association across a gene cluster. Finally, a novel algorithm is employed to evaluate potential epistatic genetic interactions to determine whether the presence of multiple polymorphisms across several biological pathways from multiple chromosomes is a factor in the development of PsA. All SNPs chosen for the studies described here were selected on the basis of having been reported in previous association studies, or because they were non-synonymous – i.e., they caused an amino acid change in the protein for which the gene codes. As described by Risch, 2000, and summarized in Table 2-1 there is an order in preference of SNPs used in genotyping studies, and we have chosen those deemed to be the highest priority for association study

## 2.4 - SNP Genotyping

The detection of SNPs was performed by the analysis of primer extension products generated from previously amplified genomic DNA using a chip-based MALDI-TOF (Matrix Assisted Laser De-ionization – Time of Flight) mass spectrometry platform (Sequenom, Inc., San Diego, CA) (308-311). PCR and extension reactions were carried out by diluting the DNA sample to be analyzed to 2.5 ng/µl, following which 1 µl of DNA was combined with 200 µM of each dNTP, 0.1 units of HotStar *Taq* (Qiagen), 0.5 µl of 10× HotStar PCR buffer containing 15 mM $MgCl_2$, PCR primers mixed together at a final concentration of 200nM (for both multiplex and uniplex reactions), and 0.2 µl of 25 mM $MgCl_2$ in a

| SNP Type | Description | Number (in thousands) |
|---|---|---|
| I | Coding, Non-synonymous, non-conservative | 60-100 |
| II | Coding, Non-synonymous, conservative | 100-180 |
| III | Coding, Synonymous | 200-240 |
| IV | Non coding, 5' UTR | 140 |
| V | Non coding, 3' UTR | 300 |
| VI | Other non-coding | ≥1000 |

**Table 2-1**: Typology of SNPs and their occurrence.

(Adapted from *Risch, 2000)*

final reaction volume of 5µl. Reactions were heated at 95°C for 15 minutes followed by 45 cycles at 95°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute and a final incubation at 72°C for 3 minute. PCR primers to amplify the region around each SNP as well as extension primers were designed using Sequenom's SpectroDesigner software. Unincorporated dNTP's were removed from the amplification products using 0.3 units of the shrimp alkaline phosphatase (Sequenom). The reaction was placed at 37°C for 20 minutes, and the enzyme was deactivated by incubating at 85°C for 5 minutes. After shrimp alkaline phosphatase treatment, the homogenous massEXTEND reaction was then performed. The massEXTEND SNP assays were designed to generate extension products of different masses by incorporating either one dideoxynucleotide or one deoxynucleotide and one dideoxynucleotide at the polymorphic site (depending on which SNP allele was present), to the antisense extension primer which annealed immediately 5′ of the polymorphic site. 1.242 µl of water, 0.2 µl of 10× Termination mix (Sequenom), 0.018 µl of 0.063 units/µl Thermosequenase (Sequenom), and 0.54 µl of 10 µM extension primer was then added to the amplification products. The MassEXTEND reaction was carried out at 94°C for 2 minutes and then 55 cycles of 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds. The reaction mix was desalted by adding 3 mg of a cationic resin, SpectroCLEAN (Sequenom), and resuspended in 16 µl of water. Reactions were carried out in 384 well plastic plates, and where possible PCR reactions were multiplexed. All post-PCR sample preparation was performed using the Multimek 96 robotic system (Beckman Coulter, Fullerton, CA). Aliquots of the

samples were spotted using a SpectroJET nanolitre dispenser (Sequenom) onto a 384-element silicon chip preloaded with a 3-hydroxypicolinic acid matrix (SpectroCHIP, Sequenom). Mass spectra were generated by the MassARRAY spectrometer (Bruker Daltonik GmbH, Bremen, Germany; SEQUENOM). Genotypes were automatically determined with the aid of the SpectroTYPER-RT software (Sequenom).

## 2.5 – Fragment Analysis

The *MICA* repeat polymorphism was genotyped using the fragment analysis module of a Beckman Coulter CEQ 8000 capillary electrophoresis automatic DNA sequencer (312). Samples were prepared for analysis by performing PCR reactions in a 96-well thin walled microplate. Amplification reactions were carried out as described in section 2.3, with the difference that cycling was as follows: 16 cycles of 94°C for 45 seconds, 60°C for 45 seconds (decreasing temperature by 0.5°C/cycle such that the first annealing temperature was 60°C while the final annealing temperature was 52°C), and 72°C for 30 seconds. This was then followed by 16 cycles of 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 30 seconds. The sample was then prepared for loading into the CEQ 8000 instrument by creating a 1:10 dilution in the CEQ Sample Loading Solution. A total of 39.5µl of this dilution was added to 0.5µl of the of the size standard for a final volume of 40.0µl. This solution is then loaded into the

instrument and the length of the fragments was calculated, and thus the number of repeats present in each sample was determined.

## 2.6 - Quality Control

Quality control measures employed to ensure that data used in the projects detailed in this thesis consisted of ensuring a 99% call rate for genotyping, as well as a 99.9% reproducibility of genotyping results. This was performed by randomly selecting 10% - 15% of samples for duplication in any PCR run to be performed in the same 384 plate as the primary reaction. Any genotypes not meeting these requirements were repeated until they met quality control criteria. Any samples which could not meet the quality control criteria were excluded from further analysis. All successful genotypes were investigated for fitness to the Hardy-Weinberg Equilibrium (HWE), and any that were not found to fit HWE were excluded.

## 2.7 – Statistical Analysis

The statistical methodologies used in this work varied depending on the nature of the specific experiment being conducted and the required analysis. Broadly, all investigated genetic variants were tested to ensure fitness to the

Hardy-Weinberg Equilibrium. The specific statistical procedures employed for each experiment (univariate/multivariate analysis, haplotyping and sliding-window haplotyping, and meta-analysis) are described in detail in the appropriate chapter pertaining to that experiment.

Statistical power was calculated according to the methodology proposed by Skol *et al.,* in Nature Genetics in 2006 (313). Assuming a sample size of 250 cases and 250 controls, a population prevalence for PsA of 0.25% as described by Gelfand *et al.,* (18), and a disease allele frequency of 0.10, we have 96% power to detect a genotype relative risk of 2.0 at a significance level of $p = 0.05$, and 55% power to detect a genotype relative risk of 1.5. With a sample size of 300 cases and 300 controls, we have 99% and 63% power to detect genotype relative risks of 2.0 and 1.5, respectively.

# 3

# Genetic Variation of the Major Histocompatibility Complex and Association with Psoriatic Arthritis

# 3.1

# TNF-α Polymorphisms and Risk of Psoriatic Arthritis in Caucasian Populations

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**Introduction:**

Psoriatic Arthritis (PsA) is a complex immunologically mediated disorder that results from interplay between multiple genetic and environmental factors (23). Although the pathogenesis of PsA is still unclear, there is a substantial contribution of genetic factors to the aetiology of PsA (163). The MHC region has long been considered to harbour underlying PsA susceptibility gene(s) and it has been estimated that at least one third of the genetic contribution of PsA resides within this region (163). The tumour necrosis factor alpha (*TNF-α*) gene, which is located 250kb centromeric from *HLA-B*, has been proposed to be a high priority candidate gene in PsA (314). This premise is supported by studies noting significantly higher levels of TNF-α in serum, synovial fluid and synovial membrane of PsA patients as compared to OA patients and healthy controls (204,315). The importance of TNF-α in PsA is further strengthened by the marked clinical response of TNF-α blockade in PsA (316). Thus TNF-α appears to be a cytokine of critical importance in PsA.

However, genetic studies of *TNF-α* polymorphisms and PsA have produced conflicting results (174,182-185,198). Selected studies have noted a significant association of TNF-α polymorphisms in PsA (182,184), while other studies have failed to detect any such association (174,183,185,198). The inconsistencies in these results may reflect to the small effect size of *TNF-α*, insufficient sample size of PsA subjects and controls, differences in populations, the presence of linkage disequilibrium, or multiple testing. In view of the location

and proposed biologic effect of TNF-α, we felt it was prudent to further evaluate the relationship between *TNF-α* promoter polymorphisms and PsA in two well characterized Canadian PsA populations, a founder population from Newfoundland and a heterogeneous population from Toronto, Ontario. This was followed by a meta-analysis of the TNF-α association studies in Caucasian PsA populations.

**Methods:**

***Patients***

This study was approved by the ethics committees at Memorial University of Newfoundland and the University of Toronto. Informed consent was obtained from all patients. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. Information was collected systematically and included age at onset of psoriasis, PsA and disease pattern. The control subjects were of similar ethnicity to the cases. Controls for the Newfoundland population were volunteers from Newfoundland who participated in our study as a result of a local campaign seeking population based controls for genetic studies. The Toronto controls were ascertained from the local HLA laboratory DNA bank which includes healthy volunteers and organ donors.

***Laboratory Method***

Blood samples were collected from patient volunteers with PsA and healthy controls in EDTA anticoagulant. DNA was extracted from peripheral blood lymphocytes using the Wizard Genomic DNA Purification Kit from Promega (Madison, WI). DNA Samples were genotyped for five TNF variants by time-of–flight mass spectrometry using the Sequenom platform. All five SNPs were in the $5^{l}$ – flanking region of TNF-α gene at the following positions: –1031 (T→C), -863 (C→A), -857 (C→T), -308 (G→A) and –238 (G→A). Primer sequences were determined using Sequenom SpectroDESIGNER software v1.3.4 (Table 3-1).

**Table 3-1: Primer sequences for TNF-α SNPs**

| SNP ID | rs number | FORWARD PRIMER | REVERSE PRIMER | MASS EXTEND PRIMER |
|---|---|---|---|---|
| -1031 | rs1799964 | 5'-ACGTTGGATGGGGAAGCAAAGGAGAAGCTG –3' | 5'-ACGTTGGATGTACATGTGGCCATATCTCCC -3' | 5'-GACCCTGACTTTTCCTTC –3' |
| -857 | rs1799724 | 5'-ACGTTGGATGCTATGGAAGTCGAGTATGGG-3' | 5'-ACGTTGGATGTATTCCATACCTGGAGGTCC –3' | 5'-CCTCTACATGGCCCTGTCTTC-3' |
| -238 | rs361525 | 5'-ACGTTGGATGACACAAATCAGTCAGTGGCC-3' | 5'-ACGTTGGATGATCAAGGATACCCCTCACAC-3' | 5'-AGAAGACCCCCCTCGGAATC-3' |
| -308 | rs1800629 | 5'-ACGTTGGATGGGTCCCCAAAAGAAATGGAG-3' | 5'-ACGTTGGATGGATTTGTGTGTAGGACCCTG-3' | 5'-GAGGCTGAACCCCGTCC-3' |
| -863 | rs1800630 | 5'-ACGTTGGATGCTATGGAAGTCGAGTATGGG-3' | 5'-ACGTTGGATGTATTCCATACCTGGAGGTCC -3' | 5'-CGAGTATGGGGACCCCC-3' |

For each sample, 2.5 ng of genomic DNA was amplified under standard conditions using the forward and reverse primer pairs. After DNA amplification, all

unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. A primer extension reaction was then carried out using the mass extend primer and the appropriate termination mix. The Primer extension products were then cleaned and spotted onto a SpectroChip. The chip was scanned using a mass spectrometry workstation (Bruker), and the resulting spectra were analyzed using the Sequenom SpectroTYPER-RT software.

With respect to the meta-analysis, the literature was searched up to and including October 2004. All articles pertaining to TNF-α polymorphisms in PsA were searched in Medline and Embase. As well the reference lists of the articles identified were also a searched. We then selected studies that examined Caucasian populations and provided enough data so that the number of minor alleles and the total number of alleles could be determined. The data was extracted by PR and then cross checked by CB.

*Statistical Analysis:*

Logistic regression was used to study the relationship between genotyping information and case/control status. The results are summarized in terms of odds ratios and significance tests. In addition an exploratory haplotype analysis based on EM imputation (317) was conducted.

Pairwise linkage disequilibrium was assessed for all combinations of TNF-α variants using Chi-squared tests. Strong associations were noted between –1031 and the other variants. The most notable association was between -863 and

–1031 ($p < 0.001$) which was present in separate populations, NF ($p < 0.001$) and Toronto ($p < 0.001$). For this reason -1031 was dropped from the haplotype analysis, which was based on the -238, -308, -857, and -863 variants. Meta-analyses were based on random effects analyses.

**Results:**

We examined 237 PsA subjects and 103 controls from Newfoundland and 203 PsA subjects and 101 controls from Toronto. The mean age of the PsA patients in Newfoundland were 50.1 years (11.3) and Toronto were 50.5 years (13.2). Forty-nine percent of PsA subjects were females in the Newfoundland cohort and 41% of PsA subjects were females in the Toronto cohort. With respect to the subtype of psoriatic arthritis, overall for both populations 61% had the polyarticular pattern, 27% the oligoarticular pattern, 5.2% the isolated spondyloarthropathy, and 7% were the remaining patterns. All probands had psoriasis vulgaris.

For the genotypes in the controls, there was no evidence of a departure from Hardy–Weinberg equilibrium (HWE) in the Newfoundland control population (*p* values were 0.13, 0.31, 0.29, 0.43, and 0.15 for 238(A), 308(A), 857(T), 863(A), and 1031(C), respectively). For the Toronto control population the comparable p values were 0.16, 0.04, 0.08, 0.24, and 0.23. While there is some weak evidence of departure from HWE for the 308(A) and possibly the 857(T) polymorphisms, this evidence would not survive any form of adjustment for multiple testing.

The genotypes and allele frequencies for PsA subjects and controls for *TNF-α* polymorphisms in the Newfoundland and Toronto population are presented in Tables 3-2 and 3-3, respectively. For completeness these tables present significance levels based on Fisher's exact test for an association between allelic frequency and disease status in the separate populations but formal comparison of these must be based on tests of heterogeneity reported subsequently.

A combined analysis of data from both populations, based on a stratified logistic regression of allelic frequencies, demonstrated a significant association between disease status and the –238(A) variant ($p = 0.01$). There was no evidence of heterogeneity of the association between the populations ($p = 0.49$). The observed frequencies in the Newfoundland PsA population versus controls for -238(A) variant were 10.7% vs. 5.3% respectively, while in the Toronto population these were 8.8% vs. 5.9%.

For the –857(T) variant, there was no evidence of a relationship with disease status based on the combined analysis ($p = 0.42$). However, there was marginal evidence of heterogeneity between the populations ($p = 0.07$). Observed allelic frequencies for the –857(T) variant in the Toronto population were 14.8% vs. 8.4% in cases and controls respectively, whereas no comparable marked difference was found in the Newfoundland population in which the frequencies were 6.8% vs. 7.8%.

**Table 3-2: *TNF-α* genotypes and minor allele frequencies of the Newfoundland Population**

| | Genotype | NF PsA patients | Controls | OR | P value |
|---|---|---|---|---|---|
| | G/G | 181 (79.4%) | 93 (90.3%) | | |
| *TNF α* -238 | G/A | 45 (19.7%) | 9 (8.7%) | | |
| | A/A | 2 (0.9%) | 1 (1.0%) | | |
| Allele F. (A) | | 10.7% | 5.3% | 2.13 | 0.03 |
| | G/G | 146 (64.9%) | 67 (65.1%) | | |
| *TNF α* -308 | G/A | 75 (33.3%) | 33 (32.0%) | | |
| | A/A | 4 (1.8%) | 3 (2.9%) | | |
| Allele F. (A) | | 18.4% | 18.9% | 0.97 | 0.91 |
| | C/C | 191 (86.8%) | 88 (85.4%) | | |
| *TNF α* -857 | T/C | 28 (12.7%) | 14 (13.6%) | | |
| | T/T | 1 (0.5%) | 1 (1.0%) | | |
| Allele F. (T) | | 6.8% | 7.8% | 0.87 | 0.74 |
| | C/C | 167 (72.3%) | 70 (68.0%) | | |
| *TNF α* -863 | A/C | 60 (26.0%) | 30 (29.1%) | | |
| | A/A | 4 (1.7%) | 3 (2.9%) | | |
| Allele F. (A) | | 14.7% | 17.5% | 0.82 | 0.36 |
| *TNF α* -1031 | T/T | 128 (58.2%) | 63 (61.2%) | | |
| | T/C | 77 (35.0%) | 33 (32.0%) | | |
| | C/C | 15 (6.8%) | 7 (6.8%) | | |
| Allele F. (C) | | 24.3% | 22.8% | 1.09 | 0.69 |

**Table 3-3: *TNF-α* Genotype and minor allele frequencies of the Toronto Population**

| | Genotype | Toronto PsA patients n=199 | Controls N=101 | OR | P value |
|---|---|---|---|---|---|
| | G/G | 164 (82.4%) | 90(89.1%) | | |
| *TNF α* -238 | G/A | 35 (17.6%) | 10(9.9%) | | |
| | A/A | 0 (0%) | 1 (1.0%) | | |
| Allele F. (A) | | 8.8% | 5.9% | 1.53 | 0.26 |
| | G/G | 144 (70.9%) | 69 (68.3%) | | |
| *TNF α* -308 | G/A | 53 (26.1%) | 25 (24.8%) | | |
| | A/A | 6 (3.0%) | 7 (6.9%) | | |
| Allele F. (A) | | 16.0% | 19.3% | 0.80 | 0.31 |
| | C/C | 148 (74.4%) | 86 (85.1%) | | |
| *TNF α* -857 | T/C | 43 (21.6%) | 13 (12.9%) | | |
| | T/T | 8 (4.0%) | 2 (2.0%) | | |
| Allele F. (T) | | 14.8% | 8.4% | 1.89 | 0.03 |
| | C/C | 155 (77.5%) | 71 (68.0%) | | |
| *TNF α* -863 | A/C | 41 (20.5%) | 26 (29.1%) | | |
| | A/A | 4 (2.0%) | 4 (2.9%) | | |
| Allele F. (A) | | 12.3% | 16.8% | 0.69 | 0.13 |
| | T/T | 126 (63%) | 61 (61.6%) | | |
| *TNF α* -1031 | T/C | 65 (32.5%) | 32 (32.3%) | | |
| | C/C | 9 (4.5%) | 6 (6.1%) | | |
| Allele F. (C) | | 20.8% | 22.2% | 0.92 | 0.67 |

For the –308(A), -863(A) and –1031(C) variants, there was no evidence of relationship with disease status (p values of 0.48, 0.28 and 0.64 respectively) or

heterogeneity of effects between populations (p values of 0.53, 0.61 and 0.57 respectively). Results based on a more conservative genotype analysis (318) not influenced by HWE assumptions (319) were very similar.

We then conducted a haplotype analysis for SNPs −238, −308, −857, and −863, in the two populations. The most notable result concerns the frequencies of the 1222 haplotype (where (1) indicates the presence of a minor allele, and (2) the presence of a major one) in patients and controls from Newfoundland ($p = 0.04$). The analysis suggests a greater frequency of this haplotype in cases than in controls. In Toronto, no significance was attached to this haplotype ($p = 0.27$). In the Toronto population the haplotype that appeared to be associated with the disease expression was 2212 ($p = 0.03$). This relation was not evident in Newfoundland ($p = 0.6$). Adjustments for multiplicity are required for interpretation purposes, however. A simple Bonferroni adjustment would generate significance levels of $0.04 \times 9 = 0.36$ and $0.03 \times 7 = 0.21$ (as we have nine possible haplotypes in Newfoundland and seven in Toronto). Thus we report these haplotype frequencies as hypothesis-generating for future studies. Nevertheless, these haplotype analyses do support the univariate SNP analyses, as they indicate that the presence of minor alleles at −238 in Newfoundland and at −857 in Toronto may be of importance for disease expression.

**Meta-analysis**

Nine cohorts from eight studies (including our two Canadian cohorts) were identified in the meta-analysis (174,182-185,198,320). The leading author, year

of publication, population, *TNF-α* variants and the number of minor and total alleles for each of the variants are listed in Table 3.1-4. The results of the meta-analysis are summarized in figure 3-1 for the *TNF-α* variants –238 and –308, which have been most extensively studied. The figures related to the other variants are not presented since they involve only three populations. Only the –238 variant was noted to have a significant association (OR 2.29 (95% CI 1.48, 3.55)). If the Japanese study is excluded from the meta-analysis, the pooled estimates for all the *TNF-α* variants were: -238(A) OR 2.37 (95% CI 1.52, 3.69); -308(A) OR 0.92 (95% CI 0.69, 1.23); -857 (T) OR 1.30 (95% CI 0.61, 2.79); -863(A) OR 0.75 (95% CI 0.55, 1.04); and -1031(C) OR 1.00 (95% CI 0.75, 1.33), the latter three being based only on the combined analysis of the Newfoundland and Toronto data. If, however, the Japanese study is included in the meta-analyses, this did not have a significant impact on the above results, leaving them practically unchanged (see fig 3.1-1 for −238(A) and −308(A)). Note that a test for heterogeneity in the –238 meta-analysis was associated with a marginal significance level of 0.05, likely associated with the higher ORs observed in the German studies (182,184). A more significant result was associated with the –308 analysis (p=0.01) due to the German_2 study's outlying OR (184). This serves as justification for the use of a mixed effects model which adjusts for heterogeneity.

## Table 3-4 – Summary of *TNF-α* associations studies in PsA

| Author | Year | Population | | -238(A) | -308(A) | -857(T) | -863(A) | -1031( C) | 488(A) |
|---|---|---|---|---|---|---|---|---|---|
| Rahman | 2005 | NFLD | Cases | 49/456 | 83/450 | 30/440 | 68/462 | 107/440 | |
| | | | | (10.7%) | (18.4%) | (6.8%) | (14.7%) | (24.3%) | |
| | | | Controls | 11/206 | 39/206 | 16/206 | 36/206 | 47/206 | |
| | | | | (5.3%) | (18.9%) | (7.8%) | (17.5%) | (22.8%) | |
| Rahman | 2005 | Toronto | Cases | 35/398 | 65/406 | 59/398 | 49/400 | 83/400 | |
| | | | | (8.8%) | (16.0%) | (14.8%) | (12.2%) | (20.8%) | |
| | | | Controls | 12/202 | 39/202 | 17/202 | 34/202 | 44/198 | |
| | | | | (5.9%) | (19.3%) | (8.4%) | (16.8%) | (22.2%) | |
| Hohler | 2002 | German_1 | Cases | 30/174 | 9/174 | | | | |
| | | | | (17.2%) | (5.2%) | | | | |
| | | | Controls | 7/198 | 34/198 | | | | |
| | | | | (3.5%) | (17.2%) | | | | |
| Hohler | 1997 | German_2 | Cases | 20/124 | 19/124 | | | | |
| | | | | (16.1%) | (15.3%) | | | | |
| | | | Controls | 7/198 | 32/198 | | | | |
| | | | | (3.5%) | (16.1%) | | | | |
| Gonzalez | 2002 | Spanish | Cases | 15/162 | 25/162 | | | | |
| | | | | (9.3%) | (15.4%) | | | | |
| | | | Controls | 14/220 | 24/220 | | | | |
| | | | | (6.4%) | (10.9%) | | | | |
| Gonzalez | 2001 | Jewish | Cases | 7/104 | 8/104 | | | | |
| | | | | (6.7%) | (7.7%) | | | | |
| | | | Controls | 3/146 | 15/146 | | | | |
| | | | | (2.0%) | (10.3%) | | | | |
| Al-Heresh | 2002 | Irish | Cases | 23/248 | 52/248 | | | | 17/248 |
| | | | | (9.3%) | (21.0%) | | | | (6.9%) |
| | | | Controls | 14/202 | 33/202 | | | | 14/202 |
| | | | | (6.9%) | (16.3%) | | | | (6.9%) |
| Balding | 2003 | Irish | Cases | | 70/298 | | | | |
| | | | | | (23.5%) | | | | |
| | | | Controls | | 172/780 | | | | |
| | | | | | (22.1%) | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Hamamoto | 2000 | Japanese | Cases | 0/40 (0%) | 0/40 (0) | 7/40 (17.5%) | 6/40 (15.0%) | 4/40 (10.0%) |
| | | | Controls | 4/174 (2.3%) | 5/174 (2.9%) | 27/174 (15.5%) | 27/174 (15.5%) | 22/174 (12.6%) |

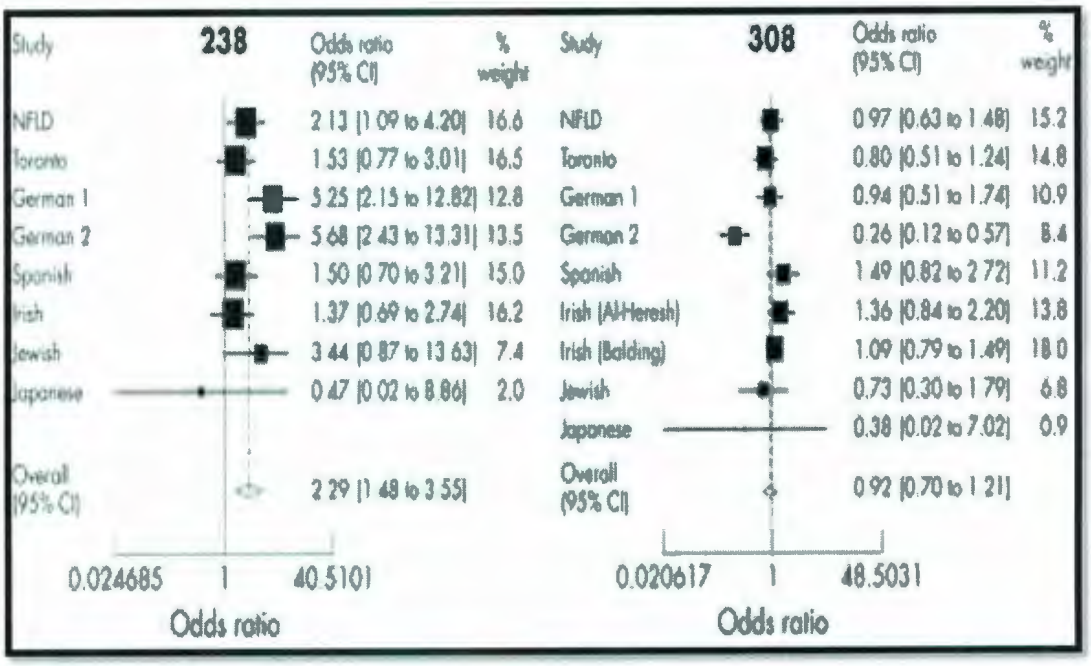


**Figure 3-1: Meta Analysis: Fixed Effects Model for *TNF-α* -238 (A) and -308 (A) variants.**

**Discussion:**

PsA has a strong heritability as reflected by a relative risk of 55 among first degree relatives with PsA (141). The *TNF-α* gene has long been considered a major susceptibility gene in immunologically mediated disease including PsA, as this cytokine is of critical importance in the pathogenesis of PsA (163). The associations between the two most widely cited *TNF-α* promoter polymorphisms (-238 and -308 variants) and PsA have been evaluated in several Caucasian populations (174,182-185,198). Prior to our study, a significant association for *TNF-α* and PsA was noted only in German populations (182,184). In this study, we examined five common variants in the promoter region of the *TNF-α* gene, including -238 and -308. We noted a significant association between -238 variant and PsA in the Newfoundland population. The magnitude of our risk (OR=2) was not as high as reported in the German studies (OR=5). This may reflect a difference in population diversity, although it is well recognized that early association studies tend to overestimate the magnitude of the association conferred by a genetic polymorphism (321).

Our pooled analysis of all *TNF-α* studies in Caucasian PsA populations also noted a significant association between –238(A) and PsA. The -238(A) variant increased the odds of PsA by two fold in Caucasian populations. The meta-analysis failed to support the association with the -308 (A) variant, which was previously reported in one of the German studies (182). The pooled estimates for the other variants revealed no significant association, although the

estimates for -857, -863 and -1031 were based only on the combined analysis of the Canadian population and the small Japanese cohort.

The differing results noted in the Irish (174,185) and Spanish (183,198) populations may be due to ethnic admixture resulting in population stratification, population specific gene-gene or gene-environment interactions, variable linkage disequilibrium between the polymorphisms and statistical fluctuations (322,323). Another possible explanation is the weak genetic effect of the underlying polymorphisms. In a meta-analysis of 370 studies addressing 36 genetic associations for various outcomes, it was noted that when the sample size of less than 150 patients were used, there is a 7 fold higher rate of discrepancy between the first and subsequent studies, as compared to studies with a sample size of at least 150 (321). Except for our studies in the Canadian cohorts, all other studies reported their finding using less than 150 PsA probands. As a result these studies may be underpowered to reliably assess the impact of *TNF-α* polymorphisms in PsA.

As the previous studies were modest in size, we conducted a meta-analysis to assist in estimating the population–wide genetic effect of the commonly cited *TNF-α* polymorphisms. Our pooled analysis demonstrated a significant association between –238(A) and PsA. Based on this finding along with the positional (i.e., close to the *HLA-Cw6* locus) and functional characteristics of *TNF-α*, we feel that there is likely a genuine association between the –238(A) variant of *TNF-α* and PsA. However, we do acknowledge

that population stratification was not entirely avoided as family based controls were not used in these studies. Furthermore, publication bias may exist in the meta-analysis, as small negative association studies are often not published. As demonstrated in our study, since meta-analysis of association studies in complex diseases are helpful in estimating population-wide genetic effects, further efforts must be made to track all association data for a given polymorphism (positive or negative, small or large, published or not).

# 3.2

# Meta-analysis of *MICA* triplet repeat polymorphism and risk of Psoriatic Arthritis in Caucasian populations

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## Introduction

Psoriatic Arthritis (PsA) is an inflammatory form of arthritis usually seronegative for rheumatoid factor (6), It may affect as many as 30% of patients with psoriasis (7,8). PsA is a complex immunologically mediated disorder which likely results from the interplay of several genetic elements and environmental factors. Although psoriasis and psoriatic arthritis (PsA) are interrelated disorders, PsA is a distinct entity with its own epidemiological clinical and genetic features. Furthermore, PsA demonstrates much greater heritability than does psoriasis among first degree relatives ($\lambda_1$ 30.4 vs. 7.6) and among siblings of PsA and psoriasis patients ($\lambda_s$ 30.8 vs. 8.8) (150).

The most common genetic association for both psoriasis and PsA has been shown to be the *HLA-Cw*0602* allele, however, other strong associations have been demonstrated for *HLA-B13*, *-B27*, *-B37*, and *–B57* (52,145). The *MICA* (major histocompatibility complex class I-related chain A) gene is a highly polymorphic gene situated in the human leukocyte antigen (HLA) class I region, located 47 kb upstream from the *HLA-B* locus, and extensive linkage disequilibrium between *HLA-B* and *MICA* has been described (187-189). The *MIC* genes represent a second lineage of mammalian MHC class I genes, and include seven members (*MICA–MICG*); five of these are pseudogenes and gene fragments, whereas *MICA* and *MICB* are functional genes closely related to each other (188). The *MICA* gene is expressed primarily in gastrointestinal epithelium (as well as keratinocytes and fibroblasts), and appears to act as a stress induced

signal for the NKG2D receptor causing activation of natural killer (NK) cells and T-cells (324,325).

*MICA* encodes a cell surface glycoprotein of 383 amino acids, composed of three extracellular domains coded for by exons 2,3 and 4, a transmembrane domain coded for by exon 5 and a carboxy-terminal cytoplasmic tail (326,327). There is a triplet microsatellite repeat in the transmembrane region of exon 5 of the *MICA* gene. These repeat alleles are named A4, A5, A6, A9, A10 reflecting 4, 5, 6, 9, or 10 GCT repeats, or A5.1 reflecting 5 GCT repeats with the insertion of an additional G nucleotide. This repeat polymorphism has been associated with several autoimmune disorders such as RA (190), Bechet's Disease (191), Ankylosing Spondylitis (192-194) and PsA (183,195-198,328), across several populations. In order to evaluate the contribution of the *MICA* repeat polymorphism we analyzed PsA patients and healthy controls from the relatively homogeneous Newfoundland population, and then performed a meta-analysis of *MICA* alleles and PsA using previously reported data.

## Methods

### *Patients*

This study was approved by the ethics committee at Memorial University of Newfoundland. Informed consent was obtained from all patients. Study participants were evaluated by a rheumatologist, and PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other

aetiologies for inflammatory arthritis. Information was collected systematically and included age at onset of psoriasis, PsA, and disease pattern. The control subjects were ascertained from the Newfoundland population, and were all unrelated.

***Laboratory Methods***

Blood samples were obtained from 309 patient volunteers with PsA and 310 healthy controls in EDTA anticoagulant blood collection tubes. DNA was extracted from peripheral blood lymphocytes using the Wizard Genomic DNA Purification Kit from Promega (Madison, WI). DNA Samples were genotyped for the *MICA* exon 5 repeat polymorphism using the Beckman Coulter CEQ 8000 automatic sequencer with primers designed using IDT's PrimerQuest program (www.idtdna.com).

With respect to the meta-analysis, the literature was searched up to and including January 2009. All articles pertaining to *MICA* repeat polymorphism in PsA were searched in Pubmed/Medline and Embase. As well, the reference lists of the articles identified were searched. We limited our selections to studies that examined Caucasian populations and provided enough data so that the number of individual repeat alleles (A4, A5, etc) as well as the total number of alleles present in both PsA patients and controls could be determined. The data was extracted by CB and then cross checked by DH and MU.

***Statistical Analysis***

Logistic regression was used to study the relationship between genotyping information and case/control status. The results are summarized in terms of odds ratios and significance tests. Pairwise linkage disequilibrium was assessed for all combinations of *MICA* repeat variants using Chi-squared tests. Meta-analyses were also based on logistic regression analyses and present results for individual studies and overall estimates of disease/genotype relationships from fixed effect stratified regression analyses.

## Results

The results of the *MICA* genotyping in the Newfoundland population is summarized in table 3-5. The *MICA* A9 allele was detected in 102/309 PsA patients vs. 71/310 control subjects (OR 1.66, 95% CI 1.16 – 2.37, $p$=0.005), and was found to be significantly associated with PsA in the Newfoundland population. *MICA* A6 was found in 79/309 patients vs. 101/310 controls (OR 0.71, 95% CI 0.50 – 1.01, $p$=0.05). For the remaining alleles, *MICA* A5.1 was observed in 226/309 vs. 218/310 (OR 1.14, 95% CI 0.81 – 1.16, $p$=0.43); for *MICA* A5 it was 51/309 vs. 72/310 (OR 0.65, 95% CI 0.44 - 0.97, $p$=0.04) and for *MICA* A-4 it was 72/309 vs. 59/310 (OR 1.29, 95% CI 0.88 – 1.90, $p$=0.19).

For the meta-analyses, a total of six studies with six different cohorts (including our own) met the inclusion criteria for analysis of the *MICA* A9 allele (195-198,328), while five were included for analysis of the *MICA* A4, A5, A6 alleles (196-198,328), and four were retained for analysis of the *MICA* A5.1 allele

**Table 3-5 – *MICA* triplet repeat polymorphism frequency in the Newfoundland population**

| MICA Repeat allele | NF PsA patients n=309 | Controls n=310 | OR (95% CI) | P value |
|---|---|---|---|---|
| A4 | 72 (23.3%) | 59 (19.0%) | 1.29 (0.88-1.90) | 0.19 |
| A5 | 51 (16.5%) | 72 (23.2%) | 0.65 (0.44-0.97) | 0.04 |
| A5.1 | 226 (73.1%) | 218 (70.3%) | 1.14 (0.81-1.16) | 0.43 |
| A6 | 79 (25.6%) | 101 (32.6%) | 0.71 (0.50-1.01) | 0.05 |
| A9 | 102 (33.0%) | 71 (22.9%) | 1.66 (1.16-2.37) | ***0.005*** |

(196-198). The meta-analysis appears to reveal a strong association between the *MICA* A9 allele and PsA.

A total of 884 PsA cases and 1023 controls from six studies were genotyped for the *MICA* A9 allele. The overall OR and 95% CI for the association between PsA and *MICA* A9 was 1.89 (1.55 - 2.31; $p < 0.0001$). There was no significant heterogeneity between these studies. For the remainder of the *MICA* alleles, the meta-analysis revealed ORs of; A4 - 1.24 (0.98 – 1.57); A5 – 0.76 (0.58 – 0.98); A5.1 – 0.91 (0.73 - 1.13); A6 – 0.79 (0.64 - 0.98) [See Figs 3.2-1 a-e).

## Figure 3-2(a-e): Meta Analysis - Fixed Effects Models for *MICA* A9, A4, A5, A5.1, and A6.

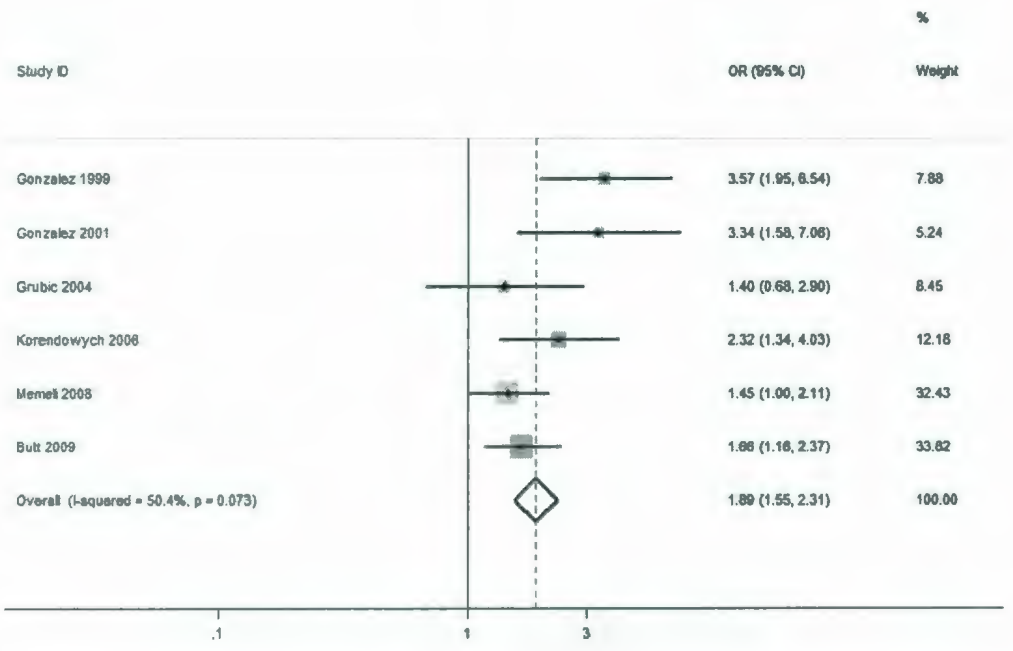


Figure 3-2a: Meta Analysis - Fixed Effects Model for *MICA* A9

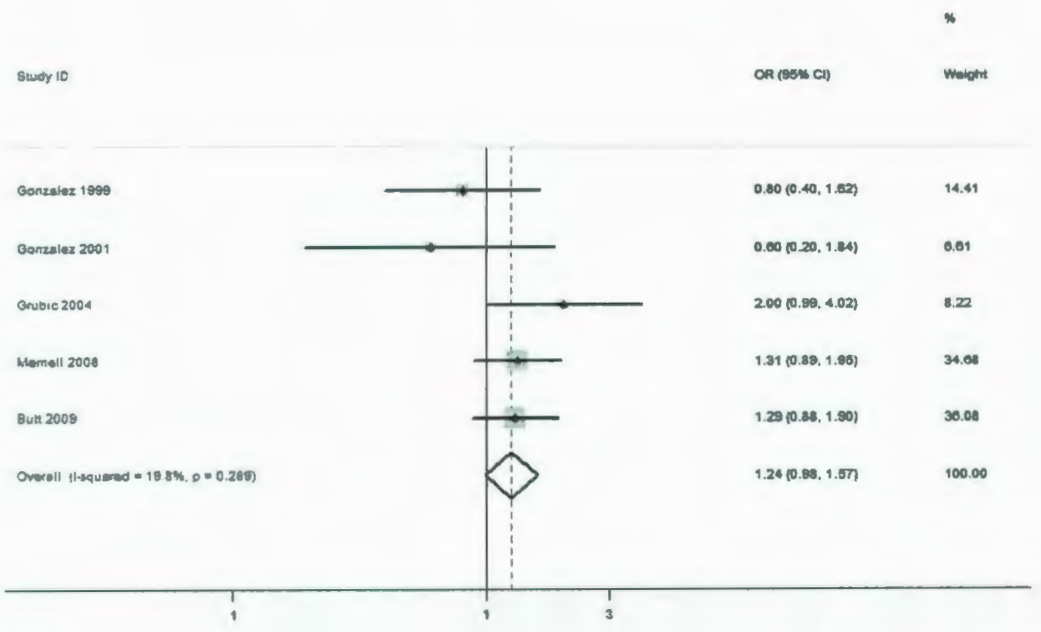


Figure 3-2b: Meta Analysis - Fixed Effects Model for *MICA* A4

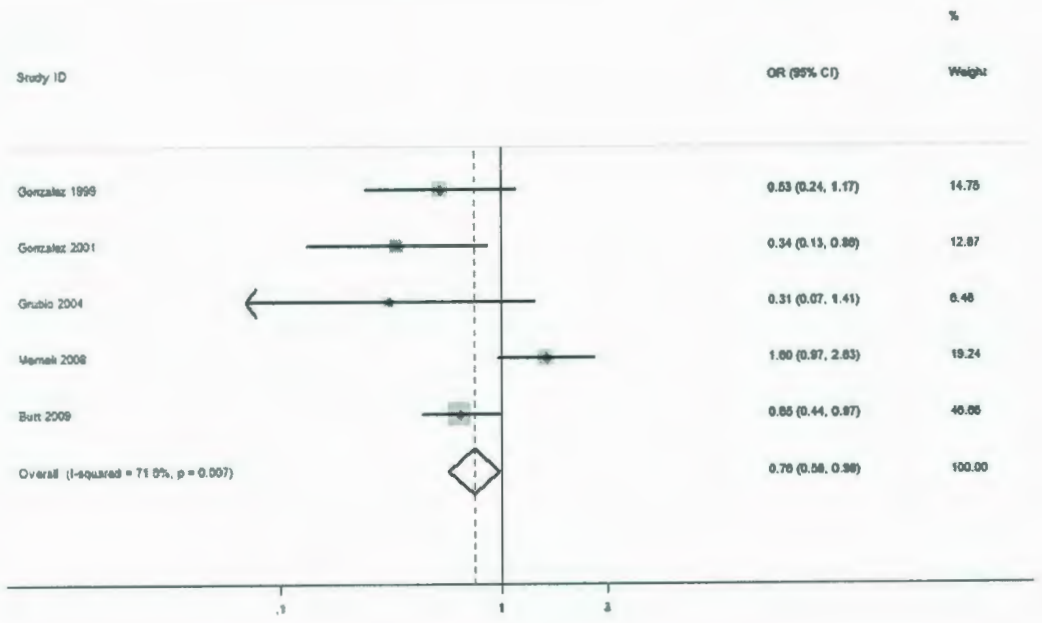


Figure 3-2c: Meta Analysis - Fixed Effects Model for *MICA* A5

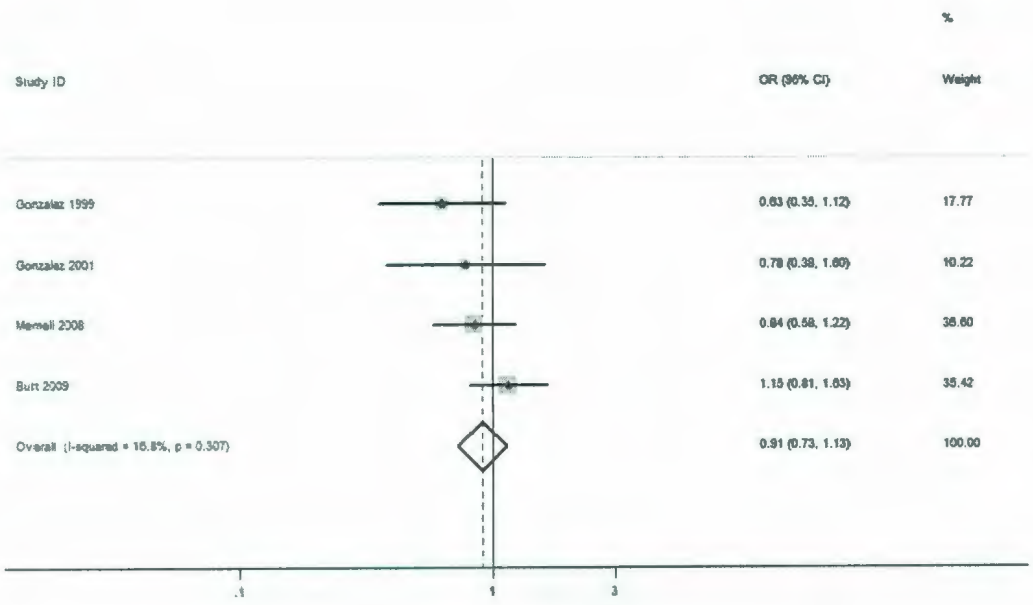


Figure 3-2d: Meta Analysis - Fixed Effects Model *MICA* A5.1

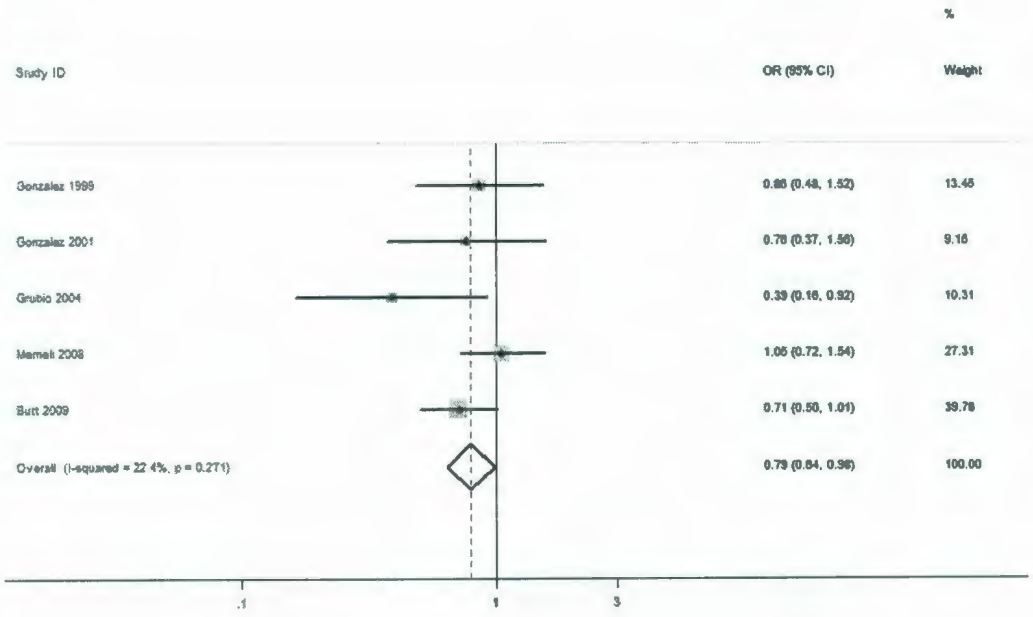


Figure 3.2-1e: Meta Analysis - Fixed Effects Model *MICA* A6

## Discussion

The strongest genetic association for both psoriasis and PsA has repeatedly been shown to be the *HLA-Cw*0602* allele, although other significant associations with *HLA-B* alleles have been demonstrated. Even in the absence of *HLA-Cw*0602,* genome wide association studies of psoriasis and PsA indicate that the strongest associations are with the MHC region (239,241). As *MICA* is located 47kb from the *HLA-B* locus, the possibility exists that *MICA* may simply be in linkage disequilibrium with a true risk allele; however, previous studies have indicated that when *MICA* alleles are associated with PsA, it is independent of *HLA-Cw*0602* (195-198). We have observed a higher prevalence of the *MICA* A9 allele in our Newfoundland PsA cohort when compared to controls (33% vs. 22.9%), and through an extensive meta-analysis have demonstrated that the presence of the *MICA* A9 allele imparts a highly significant odds ratio 1.89 (95% CI 1.55 - 2.31; $p < 0.0001$) indicating that the *MICA* A9 allele is a risk factor for PsA.

PsA is thought to be a T cell mediated disease (92,93). It has been shown that the MICA protein is recognized as a ligand by intestinal epithelium NKG2D receptors of natural killer (NK) T-cells (329), although evidence suggests that MICA is not involved in antigen presentation (330). The recognition of MICA by NKG2D receptors is interesting as it has been reported that PsA is associated with variation of another family of NK receptors, the killer immunoglobulin-like receptors (KIR) (210,213,331). It has long been recognized that patients with Crohn Disease/Ulcerative Colitis have an increased risk for the development of

PsA (263,265). There have been reported associations of *MICA* repeat polymorphisms and ulcerative colitis (332,333) as well as several other immunologically mediated diseases such as type I diabetes (IDDM) (334) and another seronegative spondyloarthropathy, ankylosing spondylitis (193,335). Cumulatively, the function of the *MICA* gene product and the reported disease associations suggest a direct involvement in PsA. Further studies exploring the functional role of *MICA* in PsA are warranted.

Finally, it is worth mentioning that upon completion of this work, another report regarding *MICA* repeat polymorphisms was released (336). While the authors did detect a higher frequency of the *MICA* A9 allele in PsA and PsA with type II psoriasis versus controls (46.0% and 58.6% vs. 30.8%, respectively) this difference did not achieve statistical significance after correction for multiple testing. However, as the sample size was small (200 PsA patients and 188 control subjects) we feel that it is unlikely these results would change the finding from our meta-analysis showing the *MICA* A9 allele is a significant risk factor for PsA.

# 3.3

# Corneodesmosin polymorphisms in psoriatic arthritis

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Based on association studies and genome wide linkage scans in psoriasis, there is convincing evidence for a major psoriasis susceptibility locus within the MHC complex [as reviewed in (163)]. The primary area of interest resides in a 200 kb region between *HLA-C* and corneodesmosin (*CDSN*) that is in strong linkage disequilibrium (LD). Despite widely reported associations between *HLA-Cw*0602* and psoriasis, there is ongoing debate about role of *HLA-Cw*0602* as the causative allele, as this allele is neither necessary nor sufficient to develop psoriasis. Meanwhile, the *CDSN* gene, which is 160 kb from *HLA-C*, has been proposed as a candidate gene in psoriasis as it is the only PSORS1 transcript to be expressed in well differentiated keratinocytes, and is responsible for corneocyte adhesion and desquamation (163). However, association studies between *CDSN* and psoriasis have reported conflicting results and the data have been difficult to interpret due to LD with *HLA-Cw6* (163,200-202,337-339). The association between *CDSN* polymorphisms and psoriatic arthritis (PsA) has not been systematically evaluated. Thus, based on the inter-relationship between psoriasis and PsA, and the location and proposed function of *CDSN*, we examined the association between *CDSN* polymorphisms and PsA in two distinct populations, a founder population from Newfoundland, Canada, and an admixed population from Toronto, Canada.

This study was approved by the local ethics committees at Memorial University of Newfoundland and the University of Toronto. Informed consent was obtained from all patients. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory

arthritis. The control subjects were ascertained from their respective regions, and were all unrelated.

Blood samples were collected from volunteers in EDTA anticoagulant and DNA was extracted from peripheral blood lymphocytes using the Wizard Genomic DNA Purification Kit from Promega (Madison, WI). The following four SNPs in the *CDSN* gene [619 (rs707913); 1215; 1236 (rs1042127); 1243 (rs3132554)], were evaluated in our study. PsA subjects and controls were genotyped for the *CDSN* polymorphism by time-of-flight mass spectrometry, using the Sequenom platform. The PCR primers were designed using MassARRAY assay design software v1.3.4. Logistic regression was used to study the relationship between genotyping information and case/control status. The results are summarized in terms of significance tests.

We examined 226 PsA subjects and 107 controls from Newfoundland and 210 PsA subjects and 99 controls from Toronto. The genotypes for the controls satisfied the Hardy-Weinberg equilibrium. The allele frequencies for PsA subjects and controls for *CDSN* polymorphisms in the Newfoundland and Ontario population are presented in Table 3-6. We observed no association between *CDSN* polymorphisms (619, 1215, 1236 and 1243), and PsA in either the Newfoundland or Ontario cohorts.

Our results contrast those of Ahnini et al, who first reported an association between *CDSN* allele (+1243) and Type I psoriasis (337) and Capon et al, who noted a cluster of three *CDSN* polymorphisms to be associated with psoriasis, in differing ethnic populations (200) as well as Schmitt-Egenolf et al. who observed

**Table 3-6:** Allele Frequencies of *CDSN* polymorphisms in Newfoundland and Toronto Population

| SNP | Newfoundland | | | Toronto | | |
|---|---|---|---|---|---|---|
| | PsA | Control | P value | PsA | Control | P value |
| 619 (C) | 35.4% | 40.6% | 0.44 | 36.3% | 32.2% | 0.58 |
| 1215 (G) | 4.9% | 1.5% | 0.37 | 8.9% | 6.2% | 057 |
| 1236 (G) | 27.4% | 30.8% | 0.61 | 38.2% | 34.3% | 0.60 |
| 1243 (T) | 70.4% | 75.2% | 0.44 | 64.5% | 69.4% | 0.48 |

that Corneodesmosin was more closely associated with psoriasis than *HLA-Cw6*0602* (338). However, the interpretation of these results is difficult as it is confounded by LD across this region. More recently, there has been heightened interest in the *CDSN* gene in psoriasis as a synonymous SNP in the *CDSN* gene has shown to increase mRNA stability and is associated with psoriasis across diverse populations (340). However, other investigators have failed to detect an association between *CDSN* polymorphisms and PsA (202,339).

In summary, we did not detect an association between *CDSN* polymorphisms and PsA in two distinct Canadian cohorts. However the possibility still remains that novel SNPs of these genes, or genes further up- or down-stream of *CDSN* may play a role in PsA. Alternatively, the *CDSN* may be in LD with the true susceptibility gene as a locus in a 70Kb interval around the *CDSN* gene has recently been mapped for psoriasis (201).

# 3.4

# Association of *SEEK1* and psoriatic arthritis in two distinct Canadian populations

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## INTRODUCTION

As psoriasis and psoriatic arthritis (PsA) are interrelated disorders, these entities, at times, share common genetic determinants (163). The association of the HLA-Cw*0602 allele with psoriasis and PsA is one such example. *HLA-Cw*0602* has long been considered to be the major predisposing factor for psoriasis (140,163), and the prevalence of this allele is also increased among subjects with PsA (341). Furthermore, in both these entities, the presence of *HLA-Cw*0602* predicts an earlier age of onset of psoriasis (157,158,174). However, whether the *HLA-Cw*0602* allele actually represents the true psoriasis gene or is in linkage disequilibrium with the true gene has not been adequately elucidated (140,163). *SEEK1* (also known as *PSORS1C1)*, which overlaps the 6p21-23 region and is in close proximity to the HLA-C locus, was recently noted to have a strong association with psoriasis in the Swedish population (199). In that study of 87 subjects with uncomplicated psoriasis, the +39604 polymorphism (rs3815097) in exon 2 of *SEEK1* was found in 66% of patients with psoriasis as compared with 22% of controls ($p = 0.000001$). This polymorphism exhibited the strongest association with psoriasis and its effect was independent of *HLA-Cw*0602*. Because of the striking association of rs3815097 in *SEEK1* and psoriasis, we set out to determine the prevalence of this *SEEK1* polymorphism in two distinct Canadian populations.

## PATIENTS AND METHODS

### Patients

This study was approved by the ethics committees at Memorial University of Newfoundland and the University of Toronto. Informed consent was obtained from all patients. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other causes for inflammatory arthritis. Information was collected systematically and included age at onset of psoriasis, PsA, and disease pattern. The control subjects were obtained from their respective regions, and were all unrelated.

### Laboratory methods

Blood samples were collected from volunteers in EDTA anticoagulant, and DNA was extracted from peripheral blood lymphocytes using the Wizard Genomic DNA Purification Kit from Promega (Madison, WI). Subjects with PsA and controls were genotyped for the *SEEK1* polymorphism by time of flight mass spectrometry, using the Sequenom platform. The polymerase chain reaction (PCR) primers were designed using MassARRAY assay design software, version 1.3.4. Mass array assay design was as follows: PCR primer 1: ACGTTGGATGTGCAACAGAAACCATCACCC; PCR primer 2: ACGTTGGATGTATGTGCTGGGAACTCAGTG. The primer sequences were obtained from Integrated DNA Technologies (Coralville, IA). For HLA genotyping,

200 ng of genomic DNA was amplified using the Dynal RELI SSO HLA-Cw* typing kit. PCR amplicons were identified by a reverse line assay using sequence-specific oligonucleotide probes. Assay results were interpreted using the pattern matching program provided by Dynal.

Statistics

Logistic regression was used to study the relationship between genotyping information and case-control status. The results are summarised as odds ratios (ORs) and significance tests. The association between age at onset of psoriasis and age at onset of PsA was examined semiparametrically (342). A $\chi^2$ test was used to examine the relationship between the *SEEK1*(T) polymorphism and disease pattern.

**RESULTS**

One hundred and three patients with PsA (42% women) and 105 ethnically matched controls from Newfoundland were assessed. The mean (SD) age of the patients with PsA from Newfoundland was 49.6 (10.7) years, mean (SD) age of onset of psoriasis was 31.0 (14.1) years and of PsA 38.0 (10.8) years. Of the 103 patients with PsA cases who were genotyped for rs3815097, there were 3 homozygotes for the mutant T allele (TT), 47 heterozygotes (TC), and 53 homozygotes for wild-type C allele (CC). Of the 105 Newfoundland controls

genotyped, there were 3 homozygotes for the mutant T allele (TT), 31 heterozygotes (TC), and 71 homozygotes for the wild-type C allele (CC). The frequency for the minor (T) allele for the rs3815097 was 48.5% in the Newfoundland patients with PsA compared with 32.4% in controls (OR = 2.0; p = 0.02). Two hundred and two patients with PsA (41% women) and 100 ethnically matched controls from Ontario were genotyped. The mean (SD) age of the patients with PsA from Ontario was 50.5 (13.2) years, mean (SD) age of onset of psoriasis was 26.9 (12.2) years and of PsA 32.5 (10.8) years. Of the 202 patients with PsA who were genotyped for rs3815097, there were 17 homozygotes for the mutant T allele (TT), 77 heterozygotes (TC), and 108 homozygotes for wild-type C allele (CC). Of the 100 Ontario controls genotyped, there were 6 homozygotes for the mutant T allele (TT), 32 heterozygotes (TC), and 62 homozygotes for the wild-type C allele (CC). The frequency for the minor (T) allele for the rs3815097 was 46.5% in the Ontario patients with PsA compared with 38.0% in controls (OR 1.4; p = 0.16). The results are summarized in table 3-7.

A combined analysis of the Newfoundland and Ontario data demonstrated an overall association between the *SEEK1*(T) allele and PsA (p = 0.009). Additionally, there was no evidence for a *SEEK1*(T) allele and geographic location interaction (p = 0.39). Thus it cannot be established that the role of the *SEEK1*(T) allele in the incidence of PsA differs in the two populations. In separate analyses, the effect of *HLA-Cw*0602* is strong in the Newfoundland population (p = 0.0004) but is not noted in the Ontario population (p = 0.41). In contrast with the *SEEK1* analysis, however, a multivariate analysis does provide evidence for an

**Table 3-7** Allele frequency of minor *SEEK1* and HLA-Cw*0602

| | Newfoundland | | | | Ontario | | | |
|---|---|---|---|---|---|---|---|---|
| | PsA | Controls | OR | p Value | PsA | Controls | OR | p Value |
| *SEEK1*(T) | 48.5 | 32.4 | 2.0 | 0.02 | 46.5 | 38.0 | 1.4 | 0.16 |
| HLA-Cw*0602 | 35.9 | 14.4 | 3.3 | 0.0004 | 28.2 | 23.5 | 1.3 | 0.41 |

interaction between *HLA-Cw*0602* and geographic location (p = 0.02), demonstrating that the effect of *HLA-Cw*0602* on the incidence of PsA is significantly different in the two populations. Furthermore, when *HLA-Cw*0602* and its interaction with location is included in a multivariate model, addition of the *SEEK1*(T) allele does not improve the model significantly (p = 0.26). Therefore, although *SEEK1*(T) is associated with PsA, *SEEK1* does not appear to be a further susceptibility factor if the *HLA-Cw*0602* status is already known. Table 1 presents a summary of the allele frequency for the minor *SEEK1* allele (T) and the presence of the *HLA-Cw*0602* allele. Linkage disequilibrium between *HLA-Cw*0602* and *SEEK1*(T) is reflected in the fact that in the Newfoundland population, 18/75 (24%) of control subjects without *HLA-Cw*0602* have the *SEEK1*(T) allele, whereas 20/23 (87%) with *HLA-Cw*0602* have the *SEEK1*(T) allele. The parallel numbers in the Ontario population are 38/107 (36%) versus 56/57 (98%). There is some evidence of a different level of disequilibrium in the

two populations (p = 0.03). When we explored the genotype/phenotype correlations for the minor (T) allele compared with the corresponding wild-type variant for *SEEK1*, no differences in the age of onset of psoriasis, PsA, or pattern of arthritis were noted.

## DISCUSSION

Presently debate exists about whether *HLA-Cw*0602* is the causative allele in psoriasis or PsA as it is neither necessary nor sufficient to develop the disease. Thus it is prudent to investigate the role of neighbouring genes, such as *SEEK1*. At first glance, rs3815097 in *SEEK1* may not appear to be a strong candidate for PsA susceptibility as the function of *SEEK1* is not yet known and rs3815097 does not code for any amino acid change. However a striking association was noted between rs3815097 in exon 2 of *SEEK1* and psoriasis in the Swedish population ($p<0.000001$) (199). This association was independent of *HLA-Cw*0602*. An efficient way to validate the importance of *SEEK1* in PsA is to study multiple case-control populations, with the expectation that any important association will be seen in all populations. We examined two complementary populations. Newfoundland is a white founder population known to exhibit homogeneity comparable to the Hutterites (306). An advantage in examining this population is that it may allow the detection of small to modest genetic associations as a result of an increased "signal to noise ratio". Meanwhile, the

Ontario population is a well characterised heterogeneous PsA population that greatly enhances the generalisability of results.

In our study a statistically significant association was shown between *SEEK1* and PsA in the Newfoundland population alone. There was a trend for increased prevalence of *SEEK1* in the Ontario PsA population as compared with controls (46.5 *v* 38.0%) but this was not significant. However, there was also no evidence for a different relationship in the two separate populations, but when both populations were combined the evidence for an association was considerable ($p = 0.009$). Further analysis showed, however, that this association was not independent of *HLA-Cw*0602* status. The association noted in the Newfoundland population can probably be attributed to the extended linkage disequilibrium that exists in this young founder population.

A puzzling finding in our study was the lack of association between *HLA-Cw*0602* and PsA in the Ontario population, especially because previous studies from this cohort have confirmed this association (46,158). The allele frequency for the Ontario controls in this study (23.5%) was higher than previously reported for Ontario controls (9–15%) (46,158). This group of controls and patients was different from groups previously reported and may highlight the heterogeneity within the Ontario population. A further limitation of our study is the inability to rule out the possibility that an association exists for other SNP variants in the *SEEK1* gene, as only one SNP variant was studied. Finally, we are unable to decipher the independent contribution of psoriasis and inflammatory arthritis to *SEEK1*, as all our patients with PsA had both psoriasis and inflammatory arthritis. In

conclusion, data from our study does not support the premise that the rs3815097 SNP in *SEEK1* is an independent genetic determinant leading to susceptibility to PsA in the Newfoundland and Ontario populations.

# 4

# Common genetic variants in auto-immune diseases: association with psoriatic arthritis.

# 4.1

# Association of *PTPN22* in psoriatic arthritis: A case-control study

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**Introduction:**

Recently, a novel gene has attracted attention in the investigation of auto-immune disease. The *PTPN22* gene encodes a functional protein tyrosine phosphatase known as lymphoid phosphatase which acts as a regulator of the negative regulatory kinase cytoplasmic tyrosine kinase (CSK) in T cells, and may play a role in suppression of T-cell activation (223). A functional SNP 1858 C>T causing an Arg→Trp substitution (R620W) which disrupts the binding site for CSK was recently found to be associated with Type-1 diabetes (IDDM) (224). Subsequently associations have also been shown with other autoimmune diseases including rheumatoid arthritis (RA) (225) and systemic lupus erythematosus (SLE) (226) in Caucasian populations. A large study in psoriasis involving 1,146 affected individuals (343) and a smaller study in psoriasis in 265 families with multiple autoimmune diseases, with only 63 psoriasis patients, revealed no association of the 1858 C>T variant of *PTPN22* and psoriasis (227).

Although psoriasis and psoriatic arthritis (PsA) are interrelated disorders, PsA is a distinct entity with its own epidemiological, clinical and genetic features. Furthermore, PsA demonstrates much greater heritability among first degree relatives ($\lambda_1$ 48) than psoriasis ($\lambda_1$ 5-10) (145). Thus we set out to examine the association of these two high priority candidate genes in two well characterized Caucasian PsA cohorts.

**Materials and Methods:**

This study was approved by the ethics committees of Memorial University of Newfoundland and University of Toronto. Informed consent was obtained from all patients. All PsA probands were Caucasians. Information was collected systematically and included age at onset of psoriasis and PsA, and disease pattern. The control subjects were of similar ethnicity to the cases. Controls for the Newfoundland population were volunteers from Newfoundland that participated in our study as a result of a local campaign seeking population based controls for genetic studies. The Toronto controls were ascertained from the local HLA laboratory DNA bank which includes healthy volunteers and organ donors.

Whole blood samples were obtained from PsA probands and control subjects. DNA was extracted using the Promega Wizard Genomic DNA purification Kit. The detection of SNPs was performed by the analysis of primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based MALDI-TOF mass spectrometry platform. In brief, PCR and extension reactions were designed using MassARRAY design software, and were carried out using 2.5 ng of template DNA. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. The amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific deoxy/dideoxy-nucleotide triphosphate (d/ddNTP) termination mixes which were also determined using MassARRAY assay design software. The primer extension products were then cleaned and spotted onto a SpectroChip. The chips were scanned using a mass spectrometry workstation

(Bruker) and the resulting spectra were analyzed and genotypes were determined using the Sequenom SpectroTYPER-RT software. We genotyped PsA probands and control subjects for the *PPTN22* 1858 C>T polymorphism (rs2476601).

To determine differences in allele and genotyping frequencies, 2 X 2 contingency tables were used. Power calculations were performed by simulating cases and controls assuming a multiplicative model for disease risk, and varying the genetic risk associated each copy of the high-risk variant. Observed allele frequencies among controls were used to generate genotypes, together with an assumed baseline risk of PsA between 0.005 and 0.01. For each candidate gene, 100 simulated datasets were created, the trend test was performed, and we counted the number of simulations where the p-value was less than 0.05.

**Results:**

Two hundred and thirty eight Newfoundland PsA patients and 149 healthy Newfoundland controls were studied. With respect to the Newfoundland PsA patients, 53% were male and their mean age at the onset of the study was 49.7 years. The mean age of onset of psoriasis was 29.3 years (sd 14.2 years) and the mean age of onset of PsA was 38.1 years (sd 11.0 years). Sixty percent of the PsA patients had polyarticular disease 32% had oligoarticular disease and 7% had an isolated spondyloarthropathy. For the Toronto population, 207 PsA patients and 203 control subjects from the Toronto population were genotyped.

With respect to the Toronto PsA patients, 61% were male and their mean age at the onset of the study was 39.6 years (sd 11.3 yrs). The mean age of onset of psoriasis was 26.8 years (sd 12.1 years) and the mean age of onset of PsA was 33.0 years (sd 10.8 years). Forty-four percent of the PsA patients had polyarticular disease, 40% had oligoarticular disease and 2.9% had isolated spondyloarthritis.

Of the 238 PsA patients genotyped for the 1858 C>T variant of *PTPN22* in the Newfoundland cohort, the C/C, C/T, and T/T genotypes for cases were 191, 44, and 3 respectively. For the 149 controls, the C/C, C/T, and T/T genotypes for controls were 121, 25, and 3 respectively. There was no difference in the minor allele (T) frequencies for the cases (10.5%) and controls (10.4%) for this *PTPN22* variant, p=0.96.

203 PsA patients and 199 control subjects were genotyped in the Toronto population for the 1858 C>T variant of *PTPN22*. For the PsA patients, the G/G, G/A, and A/A genotypes were 153, 43, and 7 respectively, while the Toronto control subjects were shown to be 167, 30, and 2. The minor allele (A) showed a frequency of 13.8% in PsA subjects versus 8.5% in controls. This was statistically significant when tested for the minor (T) allele (p=0.018) and for a trend in the genotypes (p=0.024). The rheumatoid factor (RF) was positive in 9% and 10% of the Newfoundland and Toronto cohorts respectively. After stratification for the presence of RF, we noted no association with RF positivity and the minor (T) allele for the *PTPN22* variant in either population.

All control genotypes satisfied the Hardy-Weinberg equilibrium. Using the minor allele frequency of 0.10, observed among the controls for the *PTPN22* gene, this study had over 85% power to detect a genotype relative risk of 2.0 or greater at *PTPN22* and power near 0.67 to detect a genotype relative risk of 1.75.

**Discussion:**

This is the first study to assess the association of the high priority candidate gene variant *PTPN22* 1858 C>T specifically in PsA. With respect to the Newfoundland population, our results are consistent with the reported studies in psoriasis (227,343). However, a modest association was noted between this *PPTN22* variant and PsA in the Toronto cohort. As the Toronto cohort is the first population to report a significant association between *PTPN22* and PsA and contradicts previous larger studies in psoriasis (227,343), this result should be interpreted with caution until it is independently validated in another PsA population. It is conceivable that a true association exists and this association is disease (PsA) and population (Toronto) specific. It is worthwhile to note that the lymphoid-specific phosphatase encoded by *PTPN22* is among the most powerful inhibitors of T-cell activation, thus there is a potential rationale for this association. Alternatively, a false positive association may have occurred in the Toronto PsA cohort due to population stratification. As the reported RA associations with *PTPN22* are almost exclusively with seropositive RA (225,227,344,345) we stratified our population based on seropositivity for

rheumatoid factor (RF), and found no association between the presence of RF and *PTPN22*.

**Conclusions:**

In conclusion, we obtained different results regarding the association of *PTPN22* in two PsA cohorts. A moderate association was noted in a well characterized, admixed PsA cohort, however this was not validated in a homogenous Caucasian cohort. Further studies in additional PsA populations are warranted to more definitively determine the role of *PPTN22* in PsA.

# 4.2

# The interleukin-1 family gene cluster and psoriatic arthritis.

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## Introduction

Psoriatic arthritis (PsA) is an inflammatory arthritis associated with psoriasis. The disease presents with a variety of pathogenic subtypes. While the triggers for the disease have not yet been elucidated, it has a strong genetic component (145,150). PsA is largely an inflammatory disease, and the inflammatory cytokine IL-1 is expressed at increased levels in the serum, synovial fluid and skin of PsA patients (204,346). Recently, several reports have found an association between the *IL-1* gene cluster and ankylosing spondylitis including amongst a cohort from Newfoundland (254,347). A large clinical and immunologic overlap exists between PsA and AS; thus based on the known role of IL-1 and the observed association between the *IL-1* gene cluster and AS, our laboratory examined the association of SNPs in the *IL-1* gene cluster with PsA in a cohort from Newfoundland (207).

A significant association was noted in PsA with 2 adjacent *IL1B* markers (rs1143627 and rs16944) and an *IL1F7* marker (rs3811047) in the Newfoundland PsA cohort. Using adjacent 3-marker haplotype windows two regions in the *IL-1* gene cluster (markers rs3783547, rs3783543, and rs17561 in *IL1A*, and a region near the end of *IL1B*, through *IL1F7*, *IL1F8*, and into *IL1F10*, containing markers rs3811047, rs1562304, and rs3811058) were found to be associated with PsA. While these results are interesting, findings in a small founder population may not be generalizable to larger, more admixed populations. Thus, we set out to

validate our observation of association in the *IL-1* gene cluster in an admixed population from Toronto.

**Methods**

This study was approved by the ethics committee of Memorial University of Newfoundland and the University of Toronto. Informed consent was obtained from all patients and participation was voluntary. Controls were volunteers from the Toronto area who participated in our study as a result of a local campaign seeking controls for genetics studies. Samples of whole blood were obtained from PsA probands and controls. DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). In total, Two hundred fifty PsA patients and two hundred forty seven controls were genotyped for 16 SNPs: 9 SNPs in *IL-1A*, 3 SNPs in *IL-1B*, and 2 SNPs each in *IL-1F7* and *IL-1F8*. The SNPs used and results for this study are summarized in Table 4.1. SNP determination was performed using the chip-based matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry platform (Sequenom, San Diego, CA). Briefly, polymerase chain reaction (PCR) and extension reactions were designed using MassArray design software (Sequenom). Primers were obtained from Integrated DNA Technologies (Coralville, IA). The PCR primers were used to amplify 2.5 ng of genomic DNA using standard conditions for MassArray genotyping. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase.

Amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific d/ddNTP termination mixes that were also determined using MassArray design software. The primer extension products were then cleaned and spotted onto a SpectroChip (Sequenom). The chip was scanned using a mass spectrometry workstation (Bruker Analytik, Rheinstetten, Germany), and the resulting spectra were analyzed using SpectroTyper-RT software (Sequenom). Single marker associations and 2, 3 and 4 marker sliding window haplotypes were calculated using the Plink software package (348), and 2, 3 and 4 marker sliding window haplotypes were calculated using PHASE version 2.1 (349)

**Results**

All markers tested fit Hardy-Weinberg Equilibrium. The SNP rs3917348 was excluded from analysis as it had a minor allele frequency of < 0.01. None of the remaining 15 markers tested displayed any significant associations. We estimated differences in haplotype frequencies between cases and controls for all overlapping windows of size 2, 3 or 4 markers across the region genotyped, using PHASE software, version 2.1 Parameter settings for PHASE were 300 burn-in, 300 runs, and 10000 permutations. To evaluate the number of significant results expected by chance, we permuted case and control labels and repeated the univariate 2-df tests, counting the number of significant results obtained in each permutation. Similarly, we permuted case and control labels and repeated the haplotype analyses, counting the number of significant case-control

associations within each gene. These permutation tests adjusted significance for the number of markers or windows tested. No combination of markers displayed significance following permutation testing. The results are summarized in Table 4-1.

**Table 4-1 – SNPs from the *Il-1* gene cluster and significance levels after permutations of sliding-window haplotypes.**

| SNP | Marker Number | 2-marker haplotype | *P*-Value | 3-marker haplotype | *P*-Value | 4-marker haplotype | *P*-Value |
|---|---|---|---|---|---|---|---|
| rs2856836 | 1 | 1-2 | 0.9292 | 1-2-3 | 0.9837 | 1-2-3-4 | 0.9770 |
| rs3783550 | 2 | 2-3 | 0.9948 | 2-3-4 | 0.9933 | 2-3-4-5 | 0.9708 |
| rs3783547 | 3 | 3-4 | 0.9935 | 3-4-5 | 0.9717 | 3-4-5-6 | 0.9741 |
| rs3783543 | 4 | 4-5 | 0.9108 | 4-5-6 | 0.9722 | 4-5-6-7 | 0.9555 |
| rs17561 | 5 | 5-6 | 0.9729 | 5-6-7 | 0.9542 | 5-6-7-8 | 0.9500 |
| rs1533463 | 6 | 6-7 | 0.9680 | 6-7-8 | 0.9222 | 6-7-8-9 | 0.9565 |
| rs3783526 | 7 | 7-8 | 0.9640 | 7-8-9 | 0.9801 | 7-8-9-10 | 0.7926 |
| rs1800587 | 8 | 8-9 | 0.8415 | 8-9-10 | 0.8569 | 8-9-10-11 | 0.7726 |
| rs1800794 | 9 | 9-10 | 0.8157 | 9-10-11 | 0.6812 | 9-10-11-12 | 0.3920 |
| rs1143627 | 10 | 10-11 | 0.4644 | 10-11-12 | 0.5144 | 10-11-12-13 | 0.5399 |
| rs16944 | 11 | 11-12 | 0.5228 | 11-12-13 | 0.4996 | 11-12-13-14 | 0.6467 |
| rs3811047 | 12 | 12-13 | 0.7899 | 12-13-14 | 0.8387 | 12-13-14-15 | 0.6832 |
| rs2723187 | 13 | 13-14 | 0.5931 | 13-14-15 | 0.4333 | | |
| rs1562304 | 14 | 14-15 | 0.3026 | | | | |
| rs1900287 | 15 | | | | | | |

## Discussion

Recently two reports of an autosomal recessive autoinflammatory disease caused by mutations affecting *IL1RN,* referred to as deficiency of the interleukin-1–receptor antagonist, or DIRA, have brought attention to the role of the *IL-1* gene cluster in inflammatory disease (350,351). The patients carrying mutations to *IL1RN* were described as having life-threatening systemic inflammation with skin and bone involvement, which was resolved with the administration of recombinant IL-1 receptor antagonist Anakinra (Biovitrum). The *IL1RN* gene lies just proximal to the *IL-1*gene cluster and codes for the interleukin-1 receptor antagonist. The IL1RN cytokine regulates IL-1β by binding tightly to the interleukin-1 receptor, thereby blocking access of interleukin-1 to the receptor. IL-1 and IL1RN are produced in patients with infection, trauma, or inflammatory conditions, where they compete for occupancy of the interleukin-1 receptor. Thus, it is likely that an inflammatory process may be affected by the relative amounts of interleukin-1 and interleukin-1–receptor antagonist circulating, a view which is supported by several animal models (352). Thus, the *IL-1* gene cluster is an attractive target in the investigation of an inflammatory disease such as PsA.

In this study, we set out to replicate the observed finding of association between the *IL-1* gene cluster in a PsA cohort from Newfoundland (207), using the same markers in a second PsA cohort from Toronto. Genetic studies of this gene cluster and the seronegative spondyloarthropathies have garnered considerable attention as several reports have noted an association between the *IL-1* gene cluster and AS across Caucasian (including a Newfoundland cohort)

and Asian populations (253,254,347,353,354). However, aside from the reported association in the Newfoundland population, a positive association between certain SNPs in the *IL-1* gene cluster and PsA has been published only one other time (205), however when we attempted to replicate the findings of Ravindran *et al.* in our Newfoundland PsA cohort, we observed no association with those specific polymorphisms (206). The fact that no significance was observed in single marker or 2, 3 and 4 marker sliding window haplotypes in the Toronto cohort could be due to population specific variation in our Newfoundland cohort. As the population of Newfoundland is relatively small, the reported results for both AS and PsA could be explained by a founder effect, or due to cryptic relatedness between patients of both diseases, or due to the result of positive selection or genetic drift. The repeated associations of the *IL-1* gene cluster with AS argue for this region's involvement in the seronegative spondyloarthropathies. As our Newfoundland PsA cohort has roughly 40% of patients who have been diagnosed with the spondylitis form of PsA (234) the reported association with the *IL-1* gene cluster may represent an association with spondyloarthropathy rather than PsA *per se*. Further investigation in additional PsA cohorts is warranted.

# 4.3

# Association of Nuclear Factor-κB in Psoriatic Arthritis

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Psoriatic Arthritis (PsA) is a complex immunologically mediated disorder that results from interplay between multiple genetic and environmental factors. Epidemiological studies implicate a substantive role for genetic factors in disease susceptibility and expression in PsA (23,140). Although association of PsA and alleles in the MHC region are well established, this region appears to contribute only one third of the total genetic variance to other forms of autoimmune inflammatory arthritis (355). Thus, it is prudent to investigate high priority candidate genes outside the MHC region in PsA.

Nuclear Factor-κB (NF-κB) exists as a multi-protein complex and is a pleiotropic transcription regulator that regulates key cytokines involved in regulating immune response including TNF-α and IL-1β (356). These cytokines are important in the pathogenesis of PsA (356). Recently, the first potentially functional polymorphism of the *NFKB1* gene on chromosome 4q24 was described: an ATTG ins/del polymorphism in the promoter region of the gene, which decreases promoter activity in ulcerative colitis (271). NF-κB has also been implicated in the pathogenesis of rheumatoid arthritis as the affinity for NF-κB binding in rheumatoid arthritis synovium is significantly higher than osteoarthritis synovium (357). Thus, based on the proposed function of *NFKB1* and its association with inflammatory arthritis and colitis we set out to investigate the role of this *NFKB1* promoter ins/del polymorphism as well as other SNPs in the *NFKB1* gene, and two genes in the NF-κB functional complex (*RELA* and *NFKBIA*) in subjects with PsA from our founder population.

**Methods**

Patients

This study was approved by the local ethics committee at Memorial University of Newfoundland. Informed consent was obtained from all patients, who were all from the Newfoundland population. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. The control subjects were also ascertained from Newfoundland and were all unrelated to each other, or to our patients.

Laboratory

Whole blood samples were obtained from PsA probands and control subjects. DNA was extracted using the Promega Wizard Genomic DNA purification Kit. The detection of SNPs was performed by the analysis of primer extension products generated from previously amplified genomic DNA using a chip-based MALDI-TOF mass spectrometry platform (Sequenom, Inc., San Diego, CA) (310). In brief, PCR and extension reactions were designed using MassARRAY design software, and were carried out using 2.5 ng of template DNA. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. The amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific d/ddNTP termination mixes which were also determined using MassARRAY assay design software. The primer extension products were then cleaned and spotted onto a SpectroChip. The chips were scanned using a mass spectrometry workstation

(Bruker) and the resulting spectra were analyzed and genotypes were determined using the Sequenom SpectroTYPER-RT software.

We genotyped PsA probands and control subjects for the following polymorphisms: *NFkB1*: in the promoter region, -94delATTG; and the non synonymous coding SNPs rs4648065, rs4648072, rs4648085, rs4648086, rs4648099; *RELA* promoter SNP rs11568292, coding SNP rs7116571, and 3' SNPs rs2009453, rs6591183; and *NFKBIA* promoter SNPs -410 (rs2233409), -642 (rs2233408), -673 (rs2233407), -949 (rs2233406) and 3′ SNPs 2643 (rs8904), 2758 (rs696) and 3053 (rs2273650).

Statistical Analysis

Chi-square analysis was used to test the single locus associations between SNPs in NF-κB complex and PsA. Associations between multi-locus haplotypes and case or control status were tested using the software PHASE, version 2.1 (http://www.stat.washington.edu/stephens/software.html). This software uses a haplotype reconstruction method that assesses similarity between haplotypes using arguments based in coalescent theory (349,358). A permutation test is performed to examine the similarity of the haplotype distributions between cases and controls. Since the algorithm considers haplotype similarity, the permutation test has power even when the number of haplotypes is large.

**Results**

Two hundred and twenty four PsA probands (52% males) and 88 ethnically matched controls (62% males) were studied. All subjects were Caucasian of North European descent and considered to be native Newfoundlanders. The mean age of the PsA probands was 50.0 years (SD 10.9 years), age at onset of psoriasis 29.4 years (SD 14.3 years) and age at onset of PsA 37.9 years (SD 11.3 years). All of the genotypes for the controls satisfied the Hardy-Weinberg equilibrium. Not all SNPs were successfully genotyped in every individual. The genotyping results of all non-homozygous polymorphisms are summarized in table 4-2.

With respect to single locus associations, none of the SNPs tested were found to be associated with PsA in the Newfoundland population. In particular, the allele frequency for the *NFKB1* -94delATTG was 41.7% in the cases and 41.6% in the controls (p=0.97). For the five non-synonymous coding SNPs of *NFKB1* (rs4648065, rs4648072, rs4648085, rs4648086, rs4648099), the genotypes were all homozygous for all probands and controls, with the exception of one control that was heterozygous for SNP rs4648072.

For *RELA* the 3′ SNP rs2009453 the minor allele © had a frequencies of 42.2% vs. 40.3% in cases and controls respectively (p=0.67); while for the 3' SNP rs6591183 the minor allele (A) frequencies were 44.1% vs. 38.6% in cases and controls respectively (p=0.23); The *RELA* promoter SNP rs11568292 and coding SNP rs7116571, were found to be homozygous in all PsA probands and controls, except for one patient who was heterozygous for rs11568292.

The *NFKBIA* promoter SNP -410 (rs2233409) was found to have minor allele (T) frequencies of 25.0% vs. 28.1% in cases and controls respectively (p=0.45); for SNP -673 (rs2233407), the minor allele (T) frequencies were 5.8% vs. 4.2% respectively (p=0.42); for SNP -949 (rs2233406) the minor allele (T) frequencies were 31.3% vs. 34.5% respectively (p=0.60); and finally for SNP 2578 (rs696) the minor allele (A) frequency was 28.4% vs. 30.5% in cases and controls respectively (p=0.21). For the remaining *NFKBIA* promoter SNP -642 (rs2233408) as well as the 3′ SNPs 2643 (rs8904), and 3053 (rs2273650) the genotypes were all homozygous for all probands and controls.

Haplotypes were formed with two markers for *RELA* (rs2009453 and rs6591183) and four markers for *NFKBIA* [-410 (rs2233409), -673 (rs2233407); -949 (rs2233406) and (rs696)]. No associations were found for haplotypes for *RELA* (0.37) and *NFKBIA* (0.90). We then re-analyzed all these markers as well as *NFKB1* -94delATTG for gene/gene interaction and again found no association (p=0.82).

Results were further analyzed to determine if there was any relationship between the minor allele frequency of each genotype, gender and early onset of psoriasis (defined as onset of psoriasis prior to age 40 years). No such association was observed.

**Table 4-2 – Frequency of polymorphisms in the *NFKB1*, *RELA* and *NFKBIA* genes in the Newfoundland population**

| | Genotype | NF PSA Patients | Controls | *p* Value |
|---|---|---|---|---|
| *NFkB1* - | | **n=224** | **n=83** | |
| 94delATTG | ATTG | 75(33.5%) | 26 (31.3%) | |
| rs28362491 | ATTG/DEL | 111(49.5%) | 45 (54.2%) | |
| | DEL/DEL | 38 (17.0%) | 12 (14.5%) | |
| Allele F. (del) | | 41.7% | 41.6% | NS |
| | | **n=196** | **n=88** | |
| *NFkB1* | AA | 196 (100%) | 86 (98.9%) | |
| rs4648072 | AG | | 1 (1.1%) | NS |
| *RELA* rs6591183 | | **n=194** | **n=88** | |
| | AA | 36 (18.6%) | 14 (15.9%) | |
| | AG | 99 (51.0%) | 40 (45.5%) | |
| Allele F. (A) | GG | 59 (30.4%) | 34 (38.6%) | |
| | | 44.0% | 38.6% | NS |
| *RELA* rs2009453 | | **n=193** | **n=88** | |
| | CC | 32 (16.6%) | 15 (17.0%) | |
| | CT | 99 (51.3%) | 41 (46.6%) | |
| Allele F. (C) | TT | 62 (32.1%) | 32 (36.4%) | |
| | | 42.2% | 40.3% | NS |
| *RELA* | | **n=182** | **n=71** | |
| rs11568292 | GG | 182 (100%) | 70 (98.6%) | |
| | AG | | 1 (1.4%) | NS |
| *NFKBIA* -949 | | **n=211** | **n=84** | |
| rs2233406 | C | 96 (45.5%) | 40 (47.6%) | |
| | CT | 98 (46.4%) | 30 (35.7%) | |
| | T | 17 (8.1%) | 14 (16.7%) | |
| Allele F. (T) | | 31.3% | 34.5% | NS |
| *NFKBIA* -673 | | **n=216** | **n=84** | |
| rs2233407 | A | 191 (88.4%) | 77 (91.7%) | |
| | AT | 25 (11.6%) | 27 (32.1%) | |
| | T | 0 | 0 | |
| Allele F. (T) | | 5.8% | 4.2% | NS |
| *NFKBIA* -410 | | **n=220** | **n=84** | |
| rs2233409 | C | 120 (54.5%) | 47 (56.0%) | |
| | CT | 90 (41.0%) | 27 (32.1%) | |
| | T | 10 (4.5%) | 10 (11.9%) | |
| Allele F. (T) | | 25.0% | 28.0% | NS |
| *NFKBIA* 2578 | | **n=222** | **n=88** | |
| (rs696) | A | 20 (9.0%) | 8 (9.1%) | |
| | AG | 86 (38.7%) | 37 (42.0%) | |
| | G | 116 (52.3%) | 43 (48.9%) | |
| Allele F. (A) | | 28.4% | 30.5% | NS |

## Discussion

As noted in a recent editorial, NF-κB serves as a "master switch" for the inflammatory cascade in rheumatic disease, as it is critically linked to many genes that result in synovitis such as pro-inflammatory cytokines and metalloproteinases (359). The NFKB1 protein is found most often bound with RelA to form the NF-κB complex (360). The complex is found inactive in the cytoplasm bound to an I-kappa-B inhibitory protein (NFKBIα), and is activated when the I-kappa-B kinases (IKBKA or IKBKB) phosphorylate serine residues on the NFKBIα protein, marking it for destruction via ubiquitination. The active NF-κB complex then is transported to the cell nucleus and binds DNA at kappa-B-binding motifs, thereby activating gene expression (361). Inappropriate activation of NF-κB has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, and atherosclerosis (275,362). There are no previous studies that have examined the role of NF-κB in PsA.

In our study, an association between PsA and SNPs of components of the NF-κB complex - *NFkB1*, *RELA*, and *NFKBIA* was not observed. In particular we did not detect any association between the *NFKB1* -94delATTG polymorphism and PsA. This study strongly suggests that the major *NFKB1* -94delATTG variant, which is associated with UC, is not associated with PsA. However, we cannot rule out the possibility that an association exists for other SNP variants in genes in the NF-κB pathway or that there is an association in other populations, as the Newfoundland population may not be representative of other admixed Caucasian populations (303). Furthermore, we also acknowledge that because of

our sample size we are unlikely to detect small differences in allele frequencies. Based on our observations, we conclude that there is no association between SNPs of components of the NF-κB complex - *NFkB1*, *RELA*, and *NFKBIA* in the Newfoundland population. The possibility remains that novel SNPs of these genes, or genes further up- or down-stream in the NF-κB pathway may contribute to dysfunction of the inflammatory process leading to PsA.

# 4.4

# Lack of association of *SLC22A4*, *SLC22A5, SLC9A3R1* and *RUNX1* variants in psoriatic arthritis.

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## INTRODUCTION

Psoriasis is a chronic inflammatory hyperproliferative skin disorder that affects 1-3% of the population (7). Up to a third of the patients with psoriasis may develop an inflammatory form of arthritis, referred to as psoriatic arthritis (PsA). Extensive epidemiological evidence supports the concept of PsA as a distinct entity (23). PsA is a heterogeneous disease with articular and extra-articular features. Moll and Wright described five clinical patterns of PsA, including symmetrical polyarthritis, a subtype that resembles rheumatoid arthritis (6). Various extra-articular features also occur in PsA including psoriasis (which is seen in the majority of subjects with PsA) and inflammatory bowel disease, such as Crohn disease. A significant overlap exists in the therapeutic approach for PsA, psoriasis, rheumatoid arthritis and Crohn disease including an excellent clinical response to TNF blockade. Thus there are shared epidemiological features, clinical manifestations and therapeutic strategies for these autoimmune diseases.

With respect to aetiology of these complex, immunologically mediated disorders, PsA, psoriasis, rheumatoid arthritis and Crohn disease appear to result from an interplay between multiple genetic and environmental factors. Common genetic elements to autoimmune diseases have been proposed as there is co-localization of susceptibility loci for autoimmune diseases in mouse (363) and also overlapping of susceptibility loci among autoimmune diseases in humans (364). Thus, when searching for susceptibility genes for PsA it is prudent to examine high priority candidate genes in related autoimmune disorders.

Recently, there have been major advances in the genetics of complex autoimmune diseases, with novel candidate genes being identified in psoriasis (*SLC9A3R1*) (365), rheumatoid arthritis (*SLC22A4* and *RUNX1*) (366), and Crohn disease (a haplotype of SNPs in SLC22A4 and SLC22A5) (269). As *SLC22A4*, *SLC22A5, SLC9A3R1* and *RUNX1* have a proposed role in chronic inflammatory disease, the phenotypes of which frequently overlap with PsA, we set out to examine the association of these genes with PsA in the Newfoundland founder population.

**MATERIALS AND METHODS**

This study was approved by the local ethics committee at Memorial University of Newfoundland. Informed consent was obtained from all PsA patients, who were all from the Newfoundland founder population. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. The control subjects were also ascertained from Newfoundland and were all unrelated to each other, or to our patients.

Whole blood samples were collected from PsA probands and control subjects in EDTA anticoagulant tubes and DNA was extracted from peripheral blood lymphocytes using the Wizard Genomic DNA Purification Kit from Promega (Madison, WI). In total, 259 PsA patients and 238 controls from Newfoundland were genotyped for three SNPs in *SLC22A4* (rs3792876, 1050152, rs3763112), 1

SNP in *SLC22A5* (rs2631367), 1 SNP in *SLC9A3R1* (rs734232), and 1 SNP in *RUNX1* (rs2268277) using the Sequenom MassArray platform using time-of-flight mass spectrometry (Sequenom, Inc., San Diego, CA) (310). All PCR primers and extension primers were designed using SpectroDESIGNER assay design software v1.3.4. All PCR and extension reactions were carried out using 2.5ng of template DNA. The primer extension products were then cleaned and spotted onto a SpectroChip. The chips were scanned using a mass spectrometry workstation (Bruker) and the resulting spectra were analyzed and genotypes were determined using the Sequenom SpectroTYPER-RT software. Not all samples were successfully genotyped for each SNP.

Chi square analysis was used to test the single locus associations between SNPs in our selected candidate genes and PsA. Associations between multi-locus haplotypes and case or control status were tested using the software PHASE, version 2.1 (http://www.stat.washington.edu/stephens/software.html). This software uses a haplotype reconstruction method that assesses similarity between haplotypes using arguments based in coalescent theory (349,358). A permutation test is performed to examine the similarity of the haplotype distributions between cases and controls. Since the algorithm considers haplotype similarity, the permutation test has power even when the number of haplotypes is large. Physical distances between the SNPs on the same chromosome were used in the PHASE analysis.

## RESULTS

Two hundred and fifty-nine PsA probands and 238 ethnically matched controls were studied. All subjects were Caucasian of North European descent and considered to be native Newfoundlanders. The mean age of PsA patients at the time of study was 49.67 years, SD 10.95 years, and 48.5% of subjects were females. The mean age of onset of psoriasis in our cohort was 29.27 years, SD 14.16 years. All of the genotypes for the controls satisfied the Hardy-Weinberg equilibrium. Not all SNPs were successfully genotyped in every individual.

With respect to single locus associations, none of the variants examined from *SLC22A4*, *SLC22A5, SLC9A3R1* and *RUNX1* were significantly associated with PsA in the Newfoundland population either by genotype or minor allele frequencies. These results are summarized in table 4-3. We then analyzed two marker haplotypes for markers of interest in rheumatoid arthritis [*SLC22A4* (rs3792876) and *RUNX1* (rs2268277)] and Crohn disease [*SLC22A4* (rs1050152) and *SLC22A5* (rs2631367)] and noted no association [(p=0.342), (p=0.81) respectively]. We also examined 2 marker combinations for the remaining SNPs in *SLC22A4* [(rs3792876 and rs1050152; p=0.48); (rs3792876 and rs3763112, p=0.580] as well as haplotypes for all four SNPs in *SLC22A4* on chromosome 5 (p=0.90). Finally we assessed the relationship of all 6 markers on three different chromosomes and again found noted no association (p=0.74).

**Table 4-3 – Genotype and allele frequency of *SLC9A3R1, SLC22A4, SLC22A4,* and *RUNX1* polymorphisms in the NL population.**

| | Genotype | NF PsA patients | Controls | OR (95% CI) | P value |
|---|---|---|---|---|---|
| *SLC9A3R1* (rs734232) | | | | | |
| Genotype Freq | GG | 89 (35.0%) | 74 (31.8%) | | 0.32 |
| | GA | 115 (45.3%) | 100 (42.9%) | | |
| | AA | 50 (19.7%) | 59 (25.3%) | | |
| Allele Freq (A) | | 42.3% | 46.8% | 0.84 (0.65-1.08) | 0.16 |
| *OCTN1/ SLC22A4* (rs1050152) | | | | | |
| Genotype Freq | CC | 80 (30.9%) | 75 (32.2%) | | 0.58 |
| | CT | 119 (45.9%) | 113 (48.5%) | | |
| | TT | 60 (23.2%) | 45 (19.3%) | | |
| Allele Freq (T) | | 46.1% | 43.6% | 1.11 (0.86-1.43) | 0.42 |
| *OCTN1/ SLC22A4* (rs 3763112) | | | | | |
| Genotype Freq | GG | 80 (31.6%) | 76 (31.9%) | | 0.99 |
| | GA | 131 (51.8%) | 122 (51.3%) | | |
| | AA | 42 (16.6%) | 40 (16.8%) | | |
| Allele Freq (A) | | 42.5% | 42.4% | 1.00 (0.78-1.29) | 0.99 |
| *OCTN1/ SLC22A4* (rs3792876) | | | | | |
| Genotype Freq | CC | 226 (87.6%) | 196 (84.5%) | | 0.61 |
| | CT | 31 (12.0%) | 35 (15.1%) | | |
| | TT | 1 (0.4%) | 1 (0.4%) | | |
| Allele Freq (T) | | 6.4% | 8.0% | 0.79 (0.48-1.28) | 0.34 |
| *OCTN 2/SLC22A5* (rs2631367) | | | | | |
| Genotype Freq | GG | 74 (28.7%) | 56 (24.9%) | | 0.62 |
| | GC | 115 (44.6%) | 108 (48.0%) | | |
| | CC | 69 (26.7%) | 61 (27.1%) | | |
| Allele Freq (G) | | 51.0% | 48.9% | 1.09 (0.84-1.40) | 0.52 |
| *RUNX1* (rs2268277) | | | | | |
| Genotype Freq | GG | 114 (44.4%) | 98 (42.8%) | | 0.23 |
| | GC | 116 (45.1%) | 95 (41.5%) | | |
| | CC | 27 (10.5%) | 36 (15.7%) | | |
| Allele Freq (C) | | 33.1% | 36.5% | 0.86 (0.66-1.12) | 0.27 |

## DISCUSSION

The chromosome 5q31 region is a region of interest in various forms of autoimmune disease as it contains a cytokine gene cluster that has been implicated in Crohn disease and rheumatoid arthritis (268,367). *Rioux et al.* (267) found significant association of the 5q31 region with Crohn disease using microsatellite analysis of 256 Crohn disease trios. Further analysis using TDT identified two SNPs, one in the *OCTN1* gene and one in the *OCTN2* gene as being significantly associated with Crohn disease, a finding which has since been confirmed (269). Given that Crohn disease has a pathogenesis associated with inflammation and autoimmunity, *Tokuhiro et al.* investigated the cytokine gene cluster on 5q31 to determine if an association existed between this region and rheumatoid arthritis and determined that a SNP within *SLC22A4/OCTN1* was significantly associated with rheumatoid arthritis (366). This SNP was shown to affect the expression of *SLC22A4/OCTN1* by altering the binding affinity to the transcription factor RUNX1. Notably, it has been previously shown that mutation of a RUNX1 binding site was associated with systemic lupus erythematosus (368). *Tokuhiro et al* also observed an association of an intronic *RUNX1* SNP with rheumatoid arthritis, however this association was determined to be independent of the observed *SLC22A4/OCTN1* association, rather than epistatic (366). Interestingly, the disruption of a RUNX1 binding site in the putative *PSORS2* locus on 17q25, less than three hundred bp downstream of the gene *SLC9A3R1* was recently associated with psoriasis (365).

We noted no association between the organic cation transporter genes and PsA in the Newfoundland population. This is in contrast to a recent British study that investigated 471 Caucasian PsA patients and 605 population controls for similar variants in *SLC22A4* and *SLC22A5* (369). They noted two SNPs, rs3763112 mapping to *SLC22A4* and rs2631367 mapping to *SLC22A5*, to be significantly associated with PsA (p=0.001 and p=0.007) respectively. Furthermore, a haplotype which was associated with Crohn disease (SNPs rs1050152 and rs2631367) was also strongly associated with PsA (p=0.0002) in their population. The lack of association of these SNPs and the Crohn disease haplotype in our study may reflect a difference in the PsA populations, as Newfoundland is a homogenous founder Caucasian population, while the population from Manchester is admixed. A potential benefit of studying the Newfoundland population is the possibility of an amplified genotype relative risk due to the enhanced signal to noise ratio that exists in the population as a result of genetic and relative environmental homogeneity. However, as locus homogeneity is also likely to exist in the Newfoundland population, not all genes of potential importance to PsA will be implicated in the Newfoundland population. We also cannot rule out the possibility that an association exists for other SNPs within these genes, or to other recently described organic cation transporter genes in the *IBD5* locus (*OCTN3*) (370). Furthermore, we also acknowledge that because our sample size is modest we are unlikely to detect small differences in allele frequencies.

Despite the observation that *SLC9A3R1* has a plausible biologic role in psoriasis (365), as it is expressed in polarized epithelial cells, we found no association between the rs734232 variant of *SLC9A3R1* examined and PsA in the Newfoundland PsA population. All patients with PsA in our population had psoriasis.

Thus, although there is significant epidemiological clinical and immunological overlap between PsA, psoriasis, rheumatoid arthritis and Crohn disease, we were unable to implicate *SLC22A4*, *SLC22A5,* SLC9A3R1, and RUNX1 in the aetiology of PsA in Newfoundland.

# 5

# Chasing disease pathology: gene variants from pathogenic mechanisms of psoriatic arthritis

# 5.1

# *VEGF*, *FGF1*, *FGF2* and *EGF* gene polymorphisms and psoriatic arthritis

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## Background

Psoriatic Arthritis (PsA) is an inflammatory form of arthritis usually seronegative for rheumatoid factor (6), which may affect as many as 30% of patients with psoriasis (7,8). Although psoriasis and psoriatic arthritis (PsA) are interrelated disorders, PsA is a distinct entity with its own epidemiological clinical and genetic features. Furthermore, PsA demonstrates much greater heritability among first degree relatives ($\lambda_1$ 48) than psoriasis ($\lambda_1$ 5-10) (145).

Angiogenesis appears to be a first-order event in both psoriasis and PsA (127). Abnormalities in the vascular morphology of the nail-folds of psoriasis patients without nail disease have been observed (128), as well as an increase in the number of synovial membrane blood vessels in PsA joint tissue (90). Recently, the peroxisome proliferator activated receptor-γ (PPARγ) agonist pioglitazone, which inhibits angiogenesis, has shown efficacy in the treatment of PsA in a small open-label study (132). Modest significance between a coding SNP of *PPARγ* and PsA patients has been observed (371), suggesting that angiogenesis may be an important area of investigation in PsA.

Among angiogenic factors, the cytokines vascular endothelial growth factor (*VEGF*), epidermal growth factor (*EGF*), and fibroblast growth factors 1 and 2 (*FGF1* and *FGF2*) are powerful mitogens and play a central role in the initiation of angiogenesis. *VEGF* is the only mitogen that specifically acts on endothelial cells, and has been shown to stimulate the elongation, network formation, and branching of nonproliferating endothelial cells in culture that are deprived of oxygen and nutrients. As endothelial cells in tumours are routinely exposed to

periodic or constant hypoxia, it has been proposed that *VEGF* contributes to the formation of blood vessels in tumourigenesis (372). *FGF1* has been shown to play a role in both angiogenesis and tumourigenesis (373), while *FGF2* has been shown to play a crucial role in both skin and cartilage wound healing (374,375), and in combination with another pro-angiogenic factor synergistically induces vascular networks which remain stable for more than a year even after depletion of angiogenic factors (376). *EGF* likewise is important in wound healing and plays a role in tumour growth and development (377).

High levels of the cytokine VEGF have been found in the synovial joints of both early and established rheumatoid arthritis (RA), PsA (130), AS, (378), and psoriasis plaques (379). The treatment of PsA with TNF-α inhibitors has been shown to significantly reduce levels of circulating VEGF and FGF1 (380).

Several *VEGF* polymorphisms have been associated with the development of psoriasis (379,381). The 936 T allele (rs3025039) of the *VEGF* gene, and haplotypes of SNPs at positions -2578 (rs699947), -1154 (rs1570360), -634 (rs2010963) and 936 have been associated with younger age of onset of RA in a Korean population (382). Furthermore a haplotype of SNPs at positions -2578, -1154 and -634 has been associated with disease severity for ankylosing spondylitis in the same population (383).

Both *FGF1* and *FGF2* have been observed to be upregulated in the synovial tissue of human subjects with RA (384) and significantly worsened clinical symptoms in a rat adjuvant-induced model of arthritis (385), while at least one polymorphism of the *EGF* gene (rs4444903) has been shown to significantly

affect *EGF* production (386). At the time of this report, genetic variations in the *FGF1*, *FGF2* or *EGF* genes have not been studied in either RA or the spondyloarthropathies.

Given the previously reported associations of *VEGF* in both RA and AS, and the role that *VEGF*, *FGF1*, *FGF2* and *EGF* play in angiogenesis, we examined genetic variants of each of these genes in PsA subjects from Newfoundland.

**Methods**

This study was approved by the ethics committee of Memorial University of Newfoundland. Informed consent was obtained from all patients. All PsA probands were Caucasians from the Newfoundland population. PsA was diagnosed as inflammatory arthritis in patients with psoriasis, in the absence of any other aetiology for inflammatory arthritis. Information was collected systematically and included age at onset of psoriasis, PsA and disease pattern. The ethnically matched, healthy control subjects were also obtained from the Newfoundland population, and were unrelated to the cases.

Whole blood samples were obtained from PsA probands and control subjects. DNA was extracted using the Promega Wizard Genomic DNA purification Kit. The detection of SNPs was performed by the analysis of primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based MALDI-TOF mass spectrometry platform. In brief, PCR and extension reactions were designed using MassARRAY design software, and

were carried out using 2.5 ng of template DNA. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. The amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific deoxy/dideoxy-nucleotide triphosphate (d/ddNTP) termination mixes which were also determined using MassARRAY assay design software. The primer extension products were then cleaned and spotted onto a SpectroChip. The chips were scanned using a mass spectrometry workstation (Bruker) and the resulting spectra were analyzed and genotypes were determined using the Sequenom SpectroTYPER-RT software. We genotyped PsA probands and control subjects for the following polymorphisms: in the *VEGF* gene rs3025039, rs699947, rs1570360, and rs2010963; in the *FGF1* gene rs34011; in the *FGF2* gene rs1048201; and in the *EGF* gene rs4444903, rs11568943 and rs2237051.

For 2 x 2 contingency tables of allele frequencies, Fisher's exact tests were conducted to calculate the exact p-values, and odds ratios were also calculated. As supporting exploratory analyses, genotype frequencies were also examined: for each of the 2 x 3 contingency tables of genotype frequencies, two different statistical methods that require somewhat different modeling assumptions were used to generate p-values: one was Fisher's exact test, and the other was the Cochrane-Armitage trend test, which may have more power than Fisher's exact test if a trend exists across genotype categories under the additive genetic effect model.

Haplotype estimation was conducted in several stages using two software packages (Haploview (387) and Phase v2.1 (349,358)): Haploview was first run on the markers for each multi-marker gene to identify the linkage disequilibrium structure and check to ensure that the markers were appropriate for inclusion in the haplotype estimates. Once the markers for which haplotypes were to be constructed were identified for each gene, then Haploview was re-run to identify the likely haplotypes and determine their relative frequencies. In order to predict the haplotypes for each subject, the Phase software was run on the markers used for Haploview. Haplotyping was performed on the genes *EGF* and *VEGF* only, as there was only one genotyped marker in each of *FGF1* and *FGF2*. Haplotype odds ratios for disease associations were calculated using the mixture logistic regression method proposed by Sham et al. (2004) (388), fitted by using WinBUGS 1.4 (Medical Research Council Biostatistics Unit, Cambridge) (389), which estimates Bayesian model parameters by using Markov chain Monte Carlo methods.

**Results**

Two hundred and fifty-eight PsA probands and 154 ethnically matched controls were studied. All subjects were Caucasian of North European descent and considered to be native Newfoundlanders. The mean age of PsA patients at the time of study was 49.67 years (sd 10.95 years), and 48.5% of subjects were females. The mean age of onset of psoriasis in our cohort was 29.27 years (sd

14.16 years). Not all SNPs were successfully genotyped in every individual. The results of the genotyping experiments are given in Table 5.1-1.

Chi squared ($\chi^2$) tests for a departure from the Hardy-Weinberg equilibrium were performed on each marker by Haploview. The only marker that attained statistical significance in the control sample is rs4444903 in *EGF* gene ($p < 0.01$), and this marker was removed from further analysis; all the other markers satisfied H-W equilibrium.

**Single Marker Association Tests**

The marker *VEGF* +936 (rs3025039) yielded a statistically significant result when Fisher's exact test was performed to compare allele frequencies between cases and controls (see Table 5-1). There was a higher proportion of T alleles among controls than cases (cases 11.6% vs. 16.8% in controls); Fisher's exact *p* value was = 0.042, OR for the T allele: 0.653 (95% CI: 0.434, 0.982). This finding is consistent with those of the statistical tests on the genotype frequencies (Fisher's exact test p = 0.082; Trend test p = 0.035). The marker *VEGF* -634 (rs2010963) also appears to yield a statistically significant result when Fisher's exact test was performed on the genotype frequencies (p = 0.037). However, neither the Fisher's exact test on the allele table (p = 0.74) nor the trend test on the genotype table (p = 0.71) was statistically significant. The remaining two SNPs in the *VEGF* gene, *VEGF* -1154 (rs1570360) and *VEGF* -2578 (rs699947) displayed no evidence of an association with PsA. For the *FGF1* and *FGF2* markers (rs34011

**Table 5-1: Genotype frequencies of SNPs in VEGF, FGF1, FGF2 and EGF in PsA patients and controls**

| | Genotype | NF PsA patients | | Controls | |
|---|---|---|---|---|---|
| *VEGF*+936 | CC | 200/258 | (77.5%) | 100/146 | (68.5%) |
| rs3025039 | CT | 56/258 | (21.7%) | 43/146 | (29.5%) |
| | TT | 2/258 | (0.8%) | 3/146 | (2.0%) |
| Allele Freq (T) | | 11.6% | | 16.8% | |
| *VEGF*-1154 | GG | 69/257 | (26.8%) | 35/147 | (23.8%) |
| rs1570360 | GA | 121/257 | (47.1%) | 69/147 | (46.9%) |
| | AA | 67/257 | (26.1%) | 43/147 | (29.3%) |
| Allele Freq (A) | | 49.6% | | 52.7% | |
| *VEGF*-2578 | AA | 68/257 | (26.5%) | 45/150 | (30.0%) |
| rs699947 | AC | 124/257 | (48.2%) | 70/150 | (46.7%) |
| | CC | 65/257 | (25.3%) | 35/150 | (23.3%) |
| Allele Freq (C) | | 49.4% | | 46.7% | |
| *VEGF*-634 | GG | 118/255 | (46.3%) | 64/127 | (50.4%) |
| rs2010963 | GC | 118/255 | (46.3%) | 45/127 | (35.4%) |
| | CC | 19/255 | (7.4%) | 18/127 | (14.2%) |
| Allele Freq (c) | | 30.6% | | 31.9% | |
| *FGF1* | CC | 110/242 | (45.5%) | 74/154 | (48.0%) |
| rs34011 | CT | 108/242 | (44.6%) | 64/154 | (41.6%) |
| | TT | 24/242 | (9.9%) | 16/154 | (10.4%) |
| Allele Freq (T) | | 32.2% | | 31.2% | |
| *FGF2* | CC | 167/254 | (65.7%) | 99/154 | (64.3%) |
| rs1048201 | CT | 79/254 | (31.1%) | 47/154 | (30.5%) |
| | TT | 8/254 | (3.2%) | 8/154 | (5.2%) |
| Allele Freq (T) | | 18.7% | | 20.5% | |
| *EGF* | AA | 209/248 | (84.3%) | 122/153 | (79.7%) |
| rs4444903 | GA | 4/248 | (1.6%) | 1/153 | (0.7%) |
| | GG | 35/248 | (14.1%) | 30/153 | (19.6%) |
| Allele Freq (G) | | 14.9% | | 19.9% | |
| *EGF* | GG | 78/256 | (30.5%) | 55/154 | (35.7%) |
| rs2237051 | GA | 138/256 | (53.9%) | 72/154 | (46.8%) |
| | AA | 40/256 | (15.6%) | 27/154 | (17.5%) |
| Allele Freq (A) | | 42.6% | | 40.9% | |
| *EGF* | GG | 233/243 | (95.9%) | 140/149 | (94.0%) |
| rs11568943 | GA | 10/243 | (4.1%) | 9/149 | (6.0%) |
| | AA | 0 | | 0 | |
| Allele Freq (A) | | 2.1% | | 3.0% | |

and rs1048201 respectively), there were no statistically significant findings in any of the tests ($p > 0.5$) that would suggest an association with PsA. For the two *EGF* SNPs, there was no evidence of association for either rs2237051 or rs11568943 in PsA.

**Haplotyping**

The *VEGF* marker +936 (rs3025039) is physically far from, and has low LD with, the other 3 markers in the gene (see Figure 5-1). Thus, it was decided that rs3025039 should be excluded when estimating multi-marker haplotypes within this gene, (this decision is consistent with the *VEGF* haplotyping work of Seo et al (383)) and only rs699947, rs1570360, and rs2010963 were considered for haplotyping. There are 3 main haplotypes (AAG, CGC, and CGG) at the loci rs699947, rs1570360, and rs2010963, and haplotype frequency estimates are shown in Table 5.1-2. Haploview performed $\chi^2$ tests comparing frequencies of haplotype CGC and CGG, respectively, in cases and controls. For CGC, the haplotype frequency was 0.325 in controls and 0.308 in cases, while the $\chi^2$ test gave a p value of 0.612. For CGG the haplotype frequency was 0.138 in controls and 0.186 in cases, while the $\chi^2$ test gave a p value of 0.077.

In order to calculate haplotype odds ratios (HOR), Phase v2.1 was used to make haplotype predictions for individuals. The haplotype frequencies generated by Phase and Haploview were compared and it was determined that the results from the two software packages were consistent with each other. A logistic regression model was then fitted to the case-control data to determine the odds

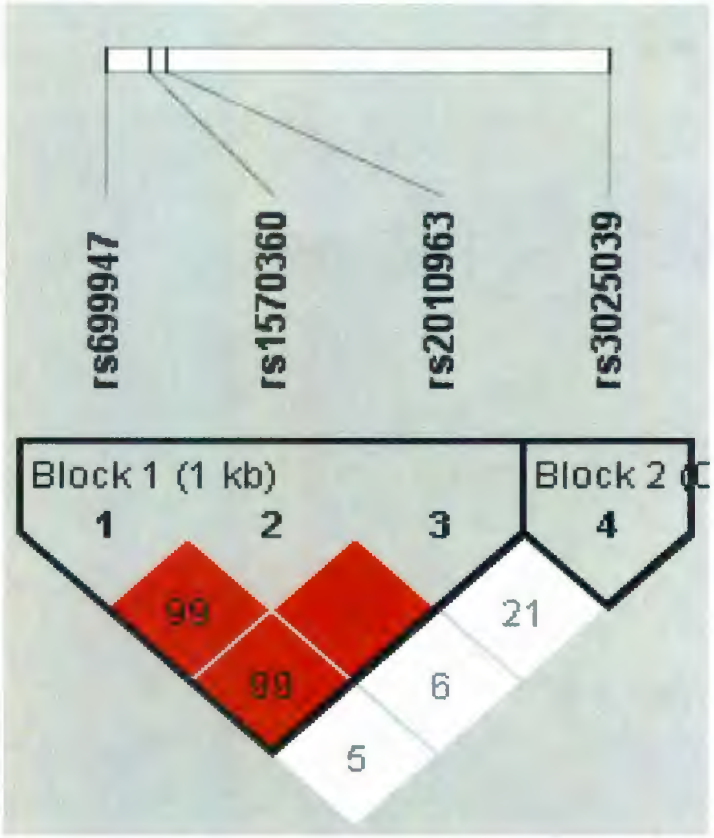


**Figure 5-1: Linkage Disequilibrium in *VEGF* Markers**

**Table 5-2: Haplotype frequencies for VEGF rs699947, rs1570360, and rs2010963, estimated by using PHASE version 2.1.**

| Haplotype | Frequency in PsA cases (N=516) No. (%) | Frequency in controls (N=300) No. (%) |
|---|---|---|
| CGC | 159.0 (30.8%) | 97.6 (32.5%) |
| CGG | 96.0 (18.6%) | 41.4 (13.8%) |
| AAG | 256.0 (49.6%) | 156.9 (52.3%) |
| All other haplotypes | 5 (0.97%) | 4.1 (1.4%) |

ratio associated with each haplotype. Since haplotypes for some individuals cannot be precisely determined due to phase ambiguities, the mixture logistic regression method (388) was used to account for the probabilistic determination of haplotypes for individuals. This was implemented in WinBUGS 1.4. Note that this implementation does not yield p-values due to its Bayesian nature, but statistical significance can still be assessed by checking whether the 95% CI of HOR spans 1.0.

The results of the HOR analysis for the *EGF* and *VEGF* markers are given in Tables 5-3 and 5-4. Neither of the haplotypes for either gene was found to be strongly related to the disease status. It was decided that the marker rs4444903 of the *EGF* gene should be excluded from haplotyping as it was not in Hardy-Weinberg equilibrium in the control sample. Thus, only *EGF* markers rs11568943 and rs2237051 were considered for haplotyping (See figure 5.1-2: The 2$^{nd}$ block of figure 1 corresponds to the haplotyping of our interest). No evidence of a

haplotype association with PsA for *EGF* markers (rs11568943 and rs2237051) was observed.

**Table 5-3: Haplotype Odds Ratios (HOR) for PsA associated with EGF rs11568943 and rs2237051. The baseline haplotype for reference is GG.**

| *EGF* haplotype | HOR (95% CI) |
|---|---|
| GA | 1.106 ( 0.824, 1.504) |
| AA | 0.702 ( 0.264, 1.844) |

**Table 5-4: Haplotype Odds Ratio (HOR) analysis for VEGF rs699947, rs1570360, and rs2010963. The reference haplotype is AAG.**

| *VEGF* haplotype | HOR (95% CI) |
|---|---|
| CGC | 0.946 ( 0.687, 1.294) |
| CGG | 1.481 ( 0.932, 2.340) |

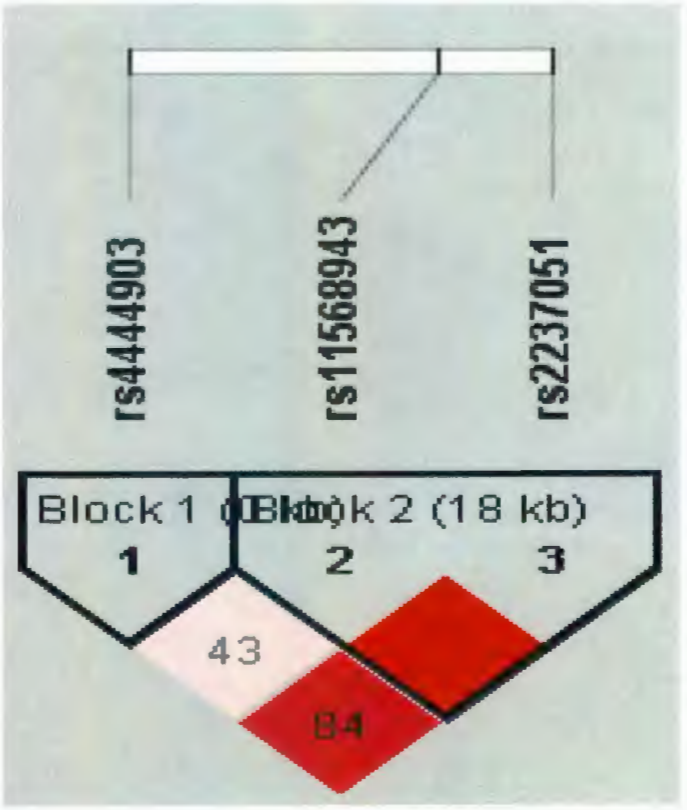


**Figure 5-2: Linkage Disequilibrium in EGF Markers**

Of the 258 PsA probands, 164 had polyarticular disease (63.6%); 79 had oligoarticular disease (30.6%); 5 patients had isolated DIP variant (1.9%), 5 had isolated spondyloarthropathies (1.9%) and 3 had arthritis mutilans (1.1%).

Due to the low frequency of the isolated DIP, isolated spondylitis, and arthritis mutilans variant, we assessed differences in allele frequencies for our markers in between the oligoarticular and polyarticular subtypes of PsA, (data summarized in Table 5-5).

With respect to axial involvement, as noted above isolated spondylitis was relatively rare in our cohort. Most of the cases of spondylitis occurred in conjunction with either oligoarthritis or polyarthritis. Thus we stratified our PsA probands as having spondylitis or no spondylitis irrespective of peripheral involvement. We noted that 40 (15.5%) of our patients had a concomitant spondyloarthropathy and 201 patients (78.0%) had no axial involvement. For the remaining 17 patients there was insufficient information to properly assess for the presence of spondylitis (data summarized in Table 5-6). As noted in tables 5.1-5 and 5.1-6, no significant differences in minor allele frequencies were noted between variants of PsA and any of the SNPs examined in our study using Fisher's exact test.

**Discussion**

The proposed physiological role of the cytokines coded for by the *VEGF*, *FGF1*, *FGF2* and *EGF* genes, along with the increased levels of these angiogen-

**Table 5-5: Minor allele frequencies in oligoarticular and polyarticular subsets of PsA.**

No significant differences were found between these two subgroups for any marker.

| SNP | Minor allele frequency for Oligoarticular disease [N= 79; 30.6% of cases] | Minor allele frequency for Polyarticular disease [N= 164; 63.6% of cases] |
|---|---|---|
| *VEGF* +936 (T) rs3025039 | 0.12 | 0.11 |
| *VEGF* -1154 (A) rs1570360 | 0.52 | 0.48 |
| *VEGF* -2578 (C) rs699947 | 0.47 | 0.51 |
| *VEGF* -634 (C) rs2010963 | 0.30 | 0.31 |
| *FGF1* (T) rs34011 | 0.32 | 0.33 |
| *FGF2* (T) rs1048201 | 0.21 | 0.18 |
| *EGF* (G) rs4444903 | 0.16 | 0.15 |
| *EGF* (A) rs2237051 | 0.46 | 0.42 |
| *EGF* (A) rs11568943 | 0.01 | 0.03 |

**Table 5.6: Minor allele frequencies in the spondyloarthritis subgroups of PsA.**

No significant differences were found between these two subgroups for any marker.

| SNP | Minor allele frequency for cases with NO spondylitis [N=201; 78.0% of cases] | Minor allele frequency for cases with Spondylitis present [N=40; 15.5% of cases] |
|---|---|---|
| VEGF +936 (T) rs3025039 | 0.12 | 0.11 |
| VEGF -1154 (A) rs1570360 | 0.48 | 0.53 |
| VEGF -2578 (C) rs699947 | 0.5 | 0.45 |
| VEGF -634 (C) rs2010963 | 0.31 | 0.29 |
| FGF1 (T) rs34011 | 0.32 | 0.36 |
| FGF2 (T) rs1048201 | 0.21 | 0.21 |
| EGF (G) rs4444903 | 0.15 | 0.20 |
| EGF (A) rs2237051 | 0.42 | 0.50 |
| EGF (A) rs11568943 | 0.02 | 0.05 |

-ic factors in arthritic synovium, make these worthy targets for evaluation of genetic association.

We have observed an increased frequency in the T allele of *VEGF* +936 (rs3025039) in control subjects when compared to our PsA patients [Fisher's exact p-value = 0.042; OR 0.653 (95% CI: 0.434, 0.982)] indicating that this SNP may play a protective role against the development of PsA. This is in contrast to Han et al. (382) who observed an association of the T allele with the development of RA. It is worth noting that the pattern of increasing vascularity in PsA synovial tissue has been shown to be markedly distinct from that observed in RA: PsA patients were shown to have predominantly tortuous, bushy vessels while RA patients had mainly straight, branching vessels (101). This potentially indicates differing mechanisms of angiogenesis, lending further evidence to the idea that there are indeed different pathogenic mechanisms in RA and PsA, which is not necessarily surprising.

Although the -1154 G→A (rs1570360) and -634 G→C (rs2010963) *VEGF* variants were not associated with rheumatoid arthritis in a white population from Spain (390), interesting observations were found in assessing patients with primary systemic vasculitides from North-western Spain. With respect to this, biopsy-proven giant cell arteritis (GCA) patients, who had severe ischemic complications exhibited a significantly increased frequency of *VEGF* -634 G allele compared with GCA patients not affected by ischemic complications or with healthy controls. Interestingly, patients carrying the *VEGF* -634 C allele, associated with high production of *VEGF* had significantly reduced frequency of

severe ischemic events in the setting of this large and middle-sized blood vessel vasculitis. In this regard, the carriage rate of the risk allele G showed statistically significant skewing comparing GCA patients with severe ischemic events with the remaining GCA patients (GG + GC compared with CC). These results suggest a potential implication of the *VEGF* gene -634 G-->C polymorphism in the development of severe ischemic manifestations of GCA. High VEGF levels may have a compensatory effect supporting neoangiogenesis mechanisms that may protect GCA patients from the development of severe ischemic complications such as irreversible visual loss (391). In contrast, in Henoch-Schonlein Purpura (HSP), a small-sized blood vessel vasculitis involving skin, gut and kidney, the high *VEGF* producing -1154 G allele was increased in HSP patients with nephritis compared with healthy controls. Similarly, the high *VEGF* producing -634 C allele was also increased in patients with nephritis compared to controls. The -1154G/-634C haplotype was also associated with susceptibility to HSP nephritis. Moreover, a protective effect against nephritis in patients with HSP was observed for the -1154A/-634G *VEGF* promoter haplotype. These results also suggest a potential implication of the VEGF -1154 G-->A and -634 G-->C polymorphisms in the development of nephritis in patients with HSP (392). It is also worth noting that in several instances the frequency of disease-associated and other alleles have been shown to be markedly different between Caucasian and Asian populations (393,394) including alleles associated with RA and other auto-immune conditions (395), therefore, our observed differences in the allele

frequency of *VEGF* +936 (rs3025039) from other published reports is not surprising.

**Conclusions**

Thus, the first investigation of genetic variations of the pro-angiogenic cytokines *VEGF*, *FGF1*, *FGF2*, and *EGF* in PsA has produced some interesting results. We observed that the T allele of *VEGF* in +936 (rs3025039) may act as a protective allele in the development of PsA, in contrast to other reports which have observed a higher frequency of the allele in RA patients. The possibility does remain that an association exists for novel SNPs in these genes which may affect transcription levels or cause other functional changes, or within regulatory genes for *VEGF*, *FGF1*, *FGF2* and *EGF*. It is also quite possible that variants in genes for other molecules which function in angiogenesis may be involved with PsA. Further studies regarding the association of pro-angiogenic markers in PsA would be beneficial to help elucidate pathogenic pathways in this disease.

# 5.2

# *PPAR-γ* gene polymorphisms and Psoriatic Arthritis

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## Introduction

Recently, Bongartz *et al.* reported on the use of a novel class of insulin-sensitizing drugs (thiazolidinediones) in the treatment of active psoriatic arthritis (PsA) (132). Specifically, in this open labelled trial, six out of 10 patients with active PsA improved sufficiently to satisfy the psoriatic arthritis response criterion (PsARC) after pioglitazone was instituted. Pioglitazone was developed for the treatment of type II diabetes mellitus and acts as a ligand for the peroxisome proliferator-activated receptor γ (*PPARγ*). The *PPARγ* gene is expressed in many human tissues and its activation has previously been shown to play a role in suppressing both angiogenesis and inflammation (133,396). Specifically, *PPARγ* activation can decrease proinflammatory cytokine expression and suppress neoangiogenesis in models of inflammatory disease (133).

*PPARγ* is a potential candidate gene for susceptibility in PsA, as Mingrone et al. demonstrated that abnormal lipid metabolism often co-exists with glucose intolerance in subjects with psoriasis (397). Secondly, epidemiological studies have noted increased prevalence of type I diabetes (IDDM) in subjects with psoriasis. Also, the Pro12Ala SNP of *PPARγ* has previously been associated with type 2 DM (398) and biomarkers of systemic inflammation (399). Finally, several animal models have shown that peroxisome proliferator-activated receptor γ agonists significantly reduced the progression of experimentally induced osteoarthritis in guinea pigs (400), and clinical disease activity scores as well as histological scores of joint destruction in a mouse model of collagen-induced arthritis (401).

Given the potential physiological role of *PPARγ* activation and the apparent success of PPARγ-agonist in the treatment of active PsA and in animal models of arthritis, we decided to examine four known coding polymorphisms in the *PPARγ* gene in our PsA population: Pro12Ala (rs1801282), Pro40Ala (rs1805192), Pro115Gln (rs1800571), and C161T (rs3856806). The PPARγ Pro12Ala SNP has previously been associated with reduced transcriptional and receptor activity of PPARγ (402) and the presence of the Ala isoform has been linked to higher insulin sensitivity and both higher and lower body mass index (403,404), while the Pro12Pro isoform has been associated with both higher levels of biomarkers of inflammation as well as shorter survival times in patients with end-stage renal disease (399). The Pro40Ala, Pro115Gln and C161T polymorphisms have all previously been inconsistently associated with diabetes, insulin sensitivity and obesity (402,404,405).

## Methods

PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. All PsA probands were Caucasians from the Newfoundland population, and were unrelated to each other. Information was collected on a standardized protocol and included age at onset of psoriasis, PsA and disease pattern. Control subjects were healthy volunteers from Newfoundland who responded to an advertisement seeking controls for genetic studies. As the control subjects were examined we verified

that they did not have PsA at the time of the study nor did they have a history of psoriasis or inflammatory arthritis. This study was approved by the local ethics committee of Memorial University of Newfoundland. Informed consent was obtained from all patients.

The island portion of the Canadian province of Newfoundland and Labrador is located in the Atlantic Ocean, off the eastern coast of Canada. The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the $17^{th}$ and $18^{th}$ centuries. The geographical and social isolation of this island has ensured very little inward migration for several hundred years and thus has lead to a small population (530 000 individuals; Statistics Canada 2001) which has grown mostly through internal expansion, with a relatively homogenous genetic background ideal for the study of complex disease.

Whole blood samples were obtained from PsA probands and control subjects. DNA was extracted using the Promega Wizard Genomic DNA purification Kit. The detection of SNPs was performed by the analysis of primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based MALDI-TOF mass spectrometry platform. In brief, PCR and extension reactions were designed using MassARRAY design software, and were carried out using 2.5 ng of template DNA. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. The amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific deoxy/dideoxy-nucleotide triphosphate (d/ddNTP)

termination mixes which were also determined using MassARRAY assay design software. The primer extension products were then cleaned and spotted onto a SpectroChip. The chips were scanned using a mass spectrometry workstation (Bruker) and the resulting spectra were analyzed and genotypes were determined using the Sequenom SpectroTYPER-RT software.

Samples were genotyped for all four *PPARγ* variants by time-of–flight mass spectrometry using the Sequenom platform. All four SNPs were previously reported coding variations, three of which caused an amino-acid change: Pro12Ala, Pro40Ala, Pro115Gln, while the fourth SNP C161T was synonymous, resulting in no amino acid change in the final protein. All primers were designed using Sequenom SpectroDESIGNER software, scanned using a mass spectrometry workstation (Bruker), and analyzed using the Sequenom SpectroTYPER-RT software. Statistical analysis of all *PPARγ* variants was performed using chi-square tests.

## Results

Of the four *PPARγ* SNPs examined, two (Pro40Ala and Pro115Gln) were found to be non-polymorphic in our population. The genotype frequencies of the remaining SNPs are given Table 5-7. For the Pro12Ala (rs1801282) SNP, an

**Table 5-7: Genotype frequencies in PPARγ in PsA patients from Newfoundland**

| | Genotype | PsA patients N=251 | Controls N=235 | p-value |
|---|---|---|---|---|
| ***PPARγ*** | CC | 208 (82.9%) | 177 (75.3%) | |
| Pro12Ala | GC | 41 (16.3%) | 51 (21.7%) | |
| rs1801282 | GG | 2 (0.8%) | 7 (3.0%) | |
| Allele Freq (G) | | 9.0% | 13.8% | 0.017 |
| ***PPARγ*** | CC | 207(80.9%) | 185 (79.4%) | |
| C1431T | TC | 43 (16.8%) | 40 (17.2%) | |
| rs3856806 | TT | 6 (2.3%) | 8 (3.4%) | |
| Allele Freq (T) | | 10.7% | 12.0% | 0.56 |

association was noted for the minor allele between PsA cases and controls (9.0% vs. 13.8% respectively, p=0.017; OR: 0.62, 95% CI: 0.45 - 0.93), and this association remained significant (p=0.034) in multiple testing using Bonferroni's correction. Meanwhile, no association was noted for the C161T (rs3856806) SNP (10.7% vs. 12.0%, respectively, p=0.56). No haplotype associations were noted and there was no correlation between the genotypes and age of onset of psoriasis, age of onset of PsA or disease pattern.

## Discussion

In our study a modest association was noted for the Pro12Ala (rs1801282) SNP, which results in an amino acid change from proline to alanine. To our knowledge, cases and controls were not related to each other. Even though selection of cases and controls was not limited to one geographic region, it is

conceivable that cryptic relatedness exists in the Newfoundland population, resulting in an ascertainment bias.

Interestingly, a previous study noted no association between PPARγ polymorphisms and uncomplicated psoriasis (406). This would appear to suggest that the Pro12Ala variant of the PPARγ gene confers susceptibility specifically to PsA rather than psoriasis. However we would use caution in interpreting this result, as 2 separate populations were studied in the psoriasis and PsA reports. Thus, further investigation of this gene from PsA and psoriasis subjects from our population as well as other independent populations is now warranted.

# 5.3

# Lack of association between genes regulating osteoclastogenesis and Psoriatic Arthritis.

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Psoriatic Arthritis (PsA) is a complex immunologically mediated disorder that results from interplay between multiple genetic and environmental factors. One of the pathological hallmarks of PsA is the presence of extensive bone resorption with concomitant new bone formation (49). Osteoclasts are the primary cells responsible for bone resorption, and there is a significant increase in the amount of osteoclast precursor cells (OPCs) observed in PsA patients (112). Signalling of OPCs to begin maturation to osteoclasts is initiated when the receptor-activator of NF-κB (RANK) – an OPC surface receptor molecule - binds to its ligand (RANKL). Osteoprotegerin (OPG) is a natural antagonist to RANKL, acting as a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting RANKL binding to RANK helping to maintain balanced bone remodelling (407). Imbalances in any of these three molecules could affect normal bone remodelling leading to the pattern of simultaneous bone loss and gain observed in PsA, thus we decided to examine some known SNPs in *RANK*, *RANKL* and *OPG* to determine if an association existed with PsA in a founder population.

This study was approved by the local ethics committee at Memorial University of Newfoundland. All patients were unrelated, and informed consent was obtained from all participants. PsA was diagnosed as an inflammatory arthritis in patients with psoriasis, in the absence of other aetiologies for inflammatory arthritis. The control subjects were ascertained from our founder population, and were all unrelated to each other and our patient group. DNA Samples were genotyped for three *OPG* SNPs (rs2073618, rs2073617,

rs3102735), two *RANKL* SNPs (rs922996, rs12721445), and one *RANK* SNP (rs1805034), by time-of–flight mass spectrometry using the Sequenom platform. All *OPG* and *RANKL* SNPs have previously reported associations with arthritic or bone-loss conditions, while the *RANK* SNP is the only identified coding SNP in that gene that results in an amino acid change present at a high heterozygosity level. All primers were designed using Sequenom SpectroDESIGNER software, scanned using a mass spectrometry workstation (Bruker), and analyzed using the Sequenom SpectroTYPER-RT software. Statistical analysis of all *OPG, RANK*, and *RANKL* variants was performed using chi-square tests.

Of the six SNPs examined, none were observed to have significant differences in minor allele frequencies between our patient and control populations (rs2073618 48.8% vs. 50.2%; rs2073617 50.0% vs. 45.5%; rs3102735 14.3% vs. 14.4%; rs1805034 48.0% vs. 51.9%; rs922996 50.0% vs. 45.1%), while rs12721445 was found to be completely non-polymorphic in our founder population (Table 5-8), and no differences were observed when analyzed by early-onset patients, gender, or type of arthritis (all $p$ values > 0.05). All genotypes satisfied the Hardy-Weinberg equilibrium.

An association between PsA and known SNPs of the genes *RANK*, *RANKL* and *OPG* was not observed in our founder population, suggesting that variants in these genes are not involved in the pathogenesis of PsA. The possibility does remain that an association exists for novel SNPs in these genes which may affect transcription levels or cause other functional changes, or that variants in genes for other molecules which play a role in osteoclast maturation or

bone resorption/formation may play a role. Furthermore, we also acknowledge that because of our sample size we are unlikely to detect small differences in allele frequencies.

Table-5-8: Association of selected SNPs in the *OPG, RANK, and RANKL* genes in PsA.

| | Genotype | # of PsA patients with each genotype | # of Controls with each genotype |
|---|---|---|---|
| *OPG* | CC | 67/252 (23.8%) | 54/238 (22.7%) |
| rs2073618 | GC | 124/252 (49.2%) | 129/238 (54.2%) |
| | GG | 61/252 (24.2%) | 55/238 (23.1%) |
| Allele Freq (G) | | 48.8% | 50.2% |
| *OPG* | TT | 61/252 (24.2%) | 67/236 (28.4%) |
| rs2073617 | CT | 130/252 (51.6%) | 123/236 (52.1%) |
| | CC | 61/252 (24.2%) | 46/236 (19.5%) |
| Allele Freq (C) | | 50.0% | 45.5% |
| *OPG* | TT | 195/259 (75.3%) | 172/236 (72.9%) |
| rs3102735 | CT | 54/259 (20.8%) | 60/236 (25.4%) |
| | CC | 10/259 (3.9%) | 4/236 (1.7%) |
| Allele Freq (C) | | 14.3% | 14.4% |
| *RANK* | TT | 76/259 (29.3%) | 50/235 (21.3%) |
| rs1805034 | CT | 117/259 (45.2%) | 126/235 (53.6%) |
| | CC | 66/259 (25.5%) | 59/235 (25.1%) |
| Allele Freq (C) | | 48.0% | 51.9% |
| *RANKL* | CC | 61/256 (23.8%) | 76/235 (32.3%) |
| rs922996 | TC | 134/256 (52.4%) | 106/235 (45.1%) |
| | TT | 61/256 (23.8%) | 53/235 (22.6%) |
| Allele Freq (T) | | 50.0% | 45.1% |
| *RANKL* | TT | 252/252 (100%) | 236/236 (100%) |
| rs12721445 | | | |

# 6

# Epistasis: a novel algorithm detects gene-gene interaction in psoriatic arthritis.

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## Introduction

The genetic contribution to complex disease is thought to be the result of multiple variants of several genes across multiple pathways, each with low penetrance (408). The predisposition imparted by individual gene variants may act independently or interact with other genes to result in an additive effect or a synergistic co-effect. As well, disease susceptibility and severity can vary across populations of differing ethnic backgrounds. This variance is likely due to the result of a shared genetic background within ethnic groups, or due to the interaction of specific gene variants with local environmental factors.

The advent of the International HapMap project (409) has enabled researchers to take advantage of knowledge of linkage disequilibrium (LD) present in the genome to elucidate the relationship between SNPs and their nearest neighbours, as well as the frequency of the these variants across several populations. The hoped-for end result is a small set of variants (tagSNPs) that capture most variation in the genome.

The data obtained from the HapMap has enabled several significant findings, from fine mapping small regions implicated in disease (221,410,411) to larger genome wide scans (239,242,412). Despite the prevailing wisdom that complex disease is the result of interplay of multiple variants, the majority of these studies do not examine potential epistatic interactions between the genotyped markers. Conventional haplotyping may not pick up associations of markers separated by great distances and thus not affected by LD on the same chromosome. Further, as multiple genes from different chromosomes interacting

to form a biological effect are the norm rather than the exception (i.e., cytokines and cytokine receptors, blood coagulation cascade) foregoing this type of intra- and intergenic marker analysis may result the underestimation of the contribution of combined gene variants to disease phenotype.

In recent years, much effort has been put into genome-wide association studies (GWAS). Frequently, such studies fail to detect associations of relevant biological pathways to disease phenotypes. One of the reasons for such failures is the use of single-locus association analyses that ignore or down-play the effects of multi-gene interactions (413). Most proposed methods and analyses for capturing epistatic effects are limited to small numbers of genes (414). One of the most prominent and commonly used methods is non-parametric multifactorial dimensionality reduction (MDR), which is a constructive induction algorithm (415). This algorithm uses 90% of the data as training data and the remaining 10% is for validation. In simulation on 10 SNPs, the MDR approach detected gene-gene interactions in the presence of 5% genotyping error and 5% missing data (416). Unfortunately, the MDR approach is limited to very small numbers of SNPs, i.e., less than 50 and is not scalable to large datasets (417,418).

Another proposed method employs multi-locus tests which use a 1 degree of freedom (1-d.f) model of interaction (419). This method has been tested successfully in simulation on 12 SNPs for two genes relative to a very small sample size and on a real dataset consisting of 6 SNPs for N-acetyltransferase 2 (*NAT 2*) relative to a moderately large case-control colorectal adenoma cohort (420). However, this method only detects interactions between two loci and does

not handle missing data. The most comprehensive method to date was proposed by Zhang et al. (421) in which they introduced a Bayesian epistasis association mapping (BEAM) model that computes the posterior probability of a disease associated marker set using Monte Carlo Markov chains. The method was successfully tested on an age-related macular degeneration genome-wide case-control data set genotyped for 96 cases and 50 controls. This method computes interactions based on six statistical models and handles interactions of up to six genes. However, it is not clear if larger groups of interactions or missing data can accommodated in this method. Common problems of existing methods are the inability to detect interactions among multiple loci, poor scalability to even moderate size datasets, and poor handling of missing data (which is very common in real datasets). The need for novel methods that solve all of these problems has been recently acknowledged as of being of great importance (422).

In order to analyze interactions such as these, we have made use of a novel algorithm developed by Mohammed Uddin (423) to examine multi-marker epistasis in PsA. The details of the algorithm and results of algorithm validation experiments are included in Appendix 1.

## Methods

### Data

The PsA cohort was taken from the Newfoundland population. Markers for analysis were chosen as SNPs from genes which we had previously reported significant associations (221,371,424,425), with additional inclusion criteria being

that the markers were bi-allelic and were hypothesized as being involved in three primary events in PsA – namely, cytokine-driven inflammation (*Il-23R*; GeneID 149233, Chromosome 1p31.3; *TNF-α*; GeneID 7124, Chromosome 6p21.3), T-cell activation (*PTPN22*; GeneID 26191, Chromosome 1p13.1), and angiogenesis (*PPAR-γ*; GeneID 5468, Chromosome 3p25).

This study was approved by the ethics committee of Memorial University of Newfoundland. Informed consent was obtained from all patients, and participation was on a purely voluntary basis. All PsA probands were Caucasians from the Newfoundland population, and were diagnosed by a rheumatologist. PsA was diagnosed as inflammatory arthritis in patients with psoriasis, in the absence of any other aetiology for inflammatory arthritis. Information was collected systematically and included age at onset of psoriasis, PsA and disease pattern. The ethnically matched, healthy control subjects were also obtained from the Newfoundland population, and were unrelated to the cases. There were a total of 240 PsA patients and 129 control subjects. The PsA case-control haplotypes includes 0.33% and 0.20% missing data, respectively

**Genotyping Methods**

Whole blood samples were obtained from PsA probands and control subjects. DNA was extracted using the Promega Wizard Genomic DNA purification kit. The detection of SNPs was performed by the analysis of primer

extension products generated from previously amplified genomic DNA using a Sequenom chip-based MALDI-TOF mass spectrometry platform (310).

## Results

The results of the analysis with our Competitive Co-Evolutionary Algorithm are displayed in Figure 6-1. After 10000 permutations, 40 SNP interactions displayed significance with p < 1.0 X 10^-4.. Of these, 22 were composed exclusively of SNPs from *Il-23R*. The highest haplotype risk ratios were observed in an AC epistatic genotype of *PPAR-γ* rs1801282 and *IL-23R* rs6660226 (HRR 2.92, 95% CI 2.04 – 4.18). The next highest HRR was observed in an AAC 'haplotype' which contained *PPAR-γ* rs1801282 and *IL-23R* rs6660226 and an additional *Il-23R* SNP of rs1857292 (HRR 2.89, 95% CI 2.02 – 4.14). *PPAR-γ* rs1801282 was observed in 18 epistatic genotypes – 6 of these included *IL-23R* rs6660226, while the remaining 12 included *Il-23R* rs11209081 (all but 1 of these 12 also included *Il-23R* rs1857292). The *PTPN22* SNP rs2476601 was only observed in three epistatic genotypes, all of which also contained *PPAR-γ* rs1801282 and *IL-23R* rs6660226. Of the 5 *TNF-α* SNPs included in this data set, only one (rs1800630, also referred to as *TNF-α* -863) was ever observed, appearing in a single CGAA epistatic genotype consisting of *TNF-α* rs1800630, *PPAR-γ* rs1801282, *IL-23R* rs6660226, *Il-23R* rs1857292 which provided the lowest observed HRR of 2.37. It remains possible that different interactions or

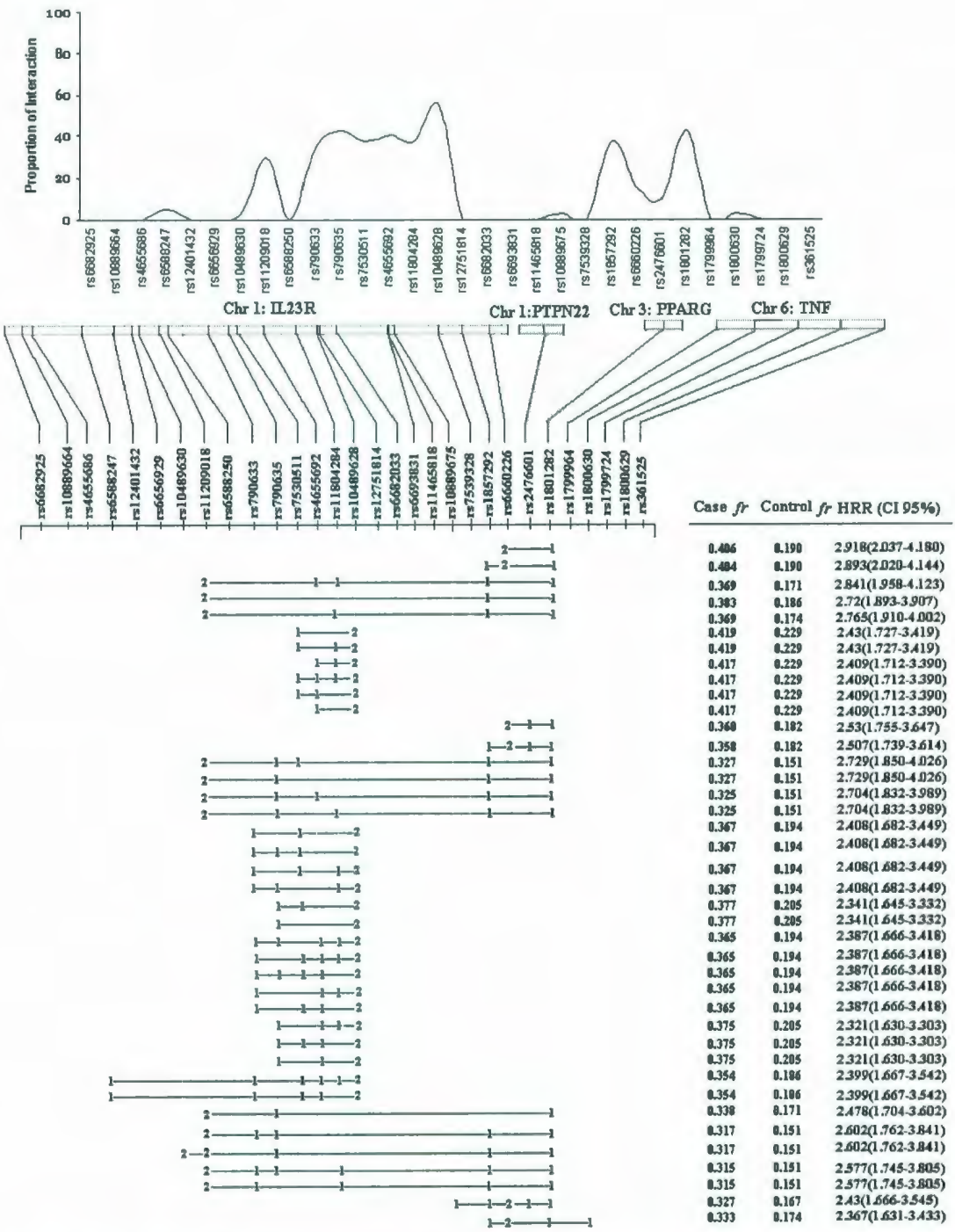


**Figure 6-1: Results of the Competitive Co-Evolutionary algorithm in PsA.**

different HRR may have been observed had our PsA patients been stratified according to the 5 different clinical subtypes (145).

**Discussion**

The results obtained here show the presence of only *IL-23R* SNPs in 22 of the 40 observed PsA epistatic genotypes and the involvement of at least one *IL-23R* SNP in all of the 40 PsA epistatic genotypes adds further evidence to the recently observed association of this gene with PsA (221,241). Functionally, IL-23R has a plausible biological role in PsA, as IL-23 is a proinflammatory cytokine and may account for pathologic T cell responses. IL-23 is also expressed on the surface of both macrophages and dendritic cells and thus may have a roll in controlling barrier function and immune response (426) IL-23 can directly induce the production of IL-22 from both murine and human naive T cells. These results suggest that Th17 cells, through the production of both IL-22 and IL-17, might have essential functions in host defence and in the pathogenesis of autoimmune diseases such as psoriasis (427).

The observation of *PPAR-γ* appearing in nearly 50% of observed significant epistatic genotypes (and always in conjunction with at least one *Il-23R* SNP) is interesting as it speaks directly to the multiple pathogenic processes (both inflammation and angiogenesis, as well as others) which must occur simultaneously in the development of PsA. *TNF-α* has been repeatedly

associated with PsA, and a recent meta-analysis of 8 populations recently showed that the -238 allele is significantly associated with PsA (424). The fact that this allele does not appear in any of our 'haplotypes' coupled with its central role in driving many inflammation processes argues that it may be an independent risk factor for PsA.

While *PTPN22* has been repeatedly associated with several immunologically mediated diseases (227) the evidence for its association with PsA has been mixed (229,425). As the gene functions in the inhibition of T cell activation, it does suggest a plausible biologic mechanism for the epistatic interactions observed here. Increased levels of the IL-23 cytokine brought about due to variation in the receptor (encoded by the *IL-23R* gene) can lead to differentiation of type 17 and type 1 helper T cells (Th17 and Th1). These activated T cells will then secrete further inflammatory cytokines which feedback into the inflammatory cycle (428). The specific *PTPN22* SNP examined here (rs2476601) introduces an Arg→Trp substitution, which disrupts a binding site for a negative regulatory kinase in T cells. Thus, a normal suppression mechanism for T cell activation is abrogated, which could further perpetuate the cycle of T-cell over-activation in psoriatic skin and joints.

It must be noted that there are some significant issues that the current iteration of our algorithm design did not consider. One such issue was the population stratification bias. Our proposed CCA algorithm is designed to detect SNPs and the underlying haplotypes from a case-control cohort that is ethnically matched. A cohort with mixed ethnicity will disrupt the accuracy of the algorithm

as mixed ethnicity will introduce a stratification bias which this algorithm does not consider while computing haplotype frequencies. Hence, using a mixed population in a cohort might produce false positive results.

Our results show the power of detecting interactions which are not restricted to a fixed number of loci, hence demonstrating the advantages of our CCA-based method over other proposed methods that use models to detect interactions from a limited number of loci. For example, while the BEAM algorithm is computationally sound, it is likely that more than 6 distinct loci can interact in complex diseases. Moreover, our CCA proposes a novel algorithm that can quickly and reliably estimate the likelihood of haplotype frequencies in the presence of up 15% missing data. This suggests that our CCA should scale well for larger datasets. There is no proposed multiple correction test that can be applied to the large number of tests we are performing in our CCA. Multiple correction tests for single locus association commonly use Bonferroni tests when all tests are considered independent. To identify gene-gene interaction from multiple loci, the LD structure can introduce dependency. Hence, the permutation test (which is applied in our CCA) is more appropriate (429). The significance level of the test was considered based on the number of permutation-iterations. As the permutation test is computationally expensive, we only apply it on haplotypes whose significance is greater than a given threshold. Further, we acknowledge that the small sample size used in each of our disease cohorts may contribute to a false-positive result. Methods of power calculation for multiple gene-gene interactions from different chromosomes are yet to be identified.

Commonly used methods (i.e. Quanto) for power computation are limited to compute for pair of genes (430). The power computation for detecting interactions from multiple genes located in different chromosome needs more research to suffice the growing need for gene-gene interactions. Finally, it is a drawback that the current iteration of the algorithm can only accommodate bi-allelic SNPs. To fully account for variation that may contribute to disease, it will be necessary to include multi-allelic SNPs, and Copy Number Variants (CNVs), such insertion/deletion or repeat polymorphisms in an analysis.

This report is meant to be a pilot study to gauge the applicability of the algorithm to single-gene and multi-gene/multi-chromosomal complex disease. As we have now shown that the model has been validated, we will soon begin to apply our software to larger datasets, and attempt to include methods for dealing more fully with other types of human genetic variation.

# 7

# Summary, Critique, and Future Directions

## 7.1 - Study Design

PsA is clearly a disease with a significant genetic component. Heritability within family units is quite high, as the recurrence risk ratio for first degree relatives of a PsA patient ($\lambda_1$) is 30.4 and for siblings ($\lambda_s$) is 30.8 (150). While it is likely a disease that involves the interaction of multiple environmental and genetic factors, identifying the genetic basis of the disease will contribute to understanding the many pathways involved in this complex disease, and help to determine an individual's susceptibility risk, or the severity of disease they are likely to experience. Establishing this would allow for earlier and more specifically targeted intervention to mitigate the effects of the disease.

Genetic studies which are designed to identify genes or polymorphisms involved with disease, or to identify genes at least in LD with a causative allele are typically carried out as either linkage studies or association studies. Linkage studies are employed when large families with multiple generations are available for analysis (431-433). Linkage analysis works well for Mendelian diseases; however, it is not as successful when used to examine complex diseases which display incomplete penetrance, genetic heterogeneity, or have multiple causative loci or an environmental trigger. In order to properly undertake linkage analysis assumptions must be made regarding such things as the mode of inheritance, disease penetrance, and candidate gene frequency. These assumptions can lead to error in the analysis if they are incorrect (144). As PsA is a complex disease, and we are have unrelated case subjects with little available family history,

linkage would be an ineffective manner in which to investigate the genetics of PsA in our patient cohort.

When large families are not available, association studies are frequently employed to evaluate genetic risk factors. Association studies are typically executed as either transmission disequilibrium tests (TDT), family based association tests (FBAT) or case-control studies. The TDT is an application of the McNamara's test statistic which measures the over-transmission of an allele from heterozygous parents to affected offspring. It requires trios consisting of parents and an affected offspring, but can also be used if there is more than one affected offspring (434). Variations have been devised which require the genotyping of only one parent (435). Compared with conventional tests for linkage, the TDT has the advantage that it does not require data either on multiple affected family members or on unaffected sibs.

The FBAT allows analysis of possibly incomplete nuclear families. (435) This test conceptualises TDT tests as a two stage procedure. In the first stage, a statistic to test for association between the trait locus and the marker locus is specified. In the second stage, the distribution of the genotyped marker data is computed by treating the offspring genotype data as random. The first stage allows flexibility in modeling while the second stage ensures correct false positive rates regardless of population admixture, genetic model misspecification or the ascertainment strategy (435). Our PsA patient cohorts consist of unrelated individual probands with no additional family members. Thus, as with traditional linkage, the TDT and FBAT would be inappropriate to analyse our subjects.

Genetic case-control association studies use subjects who already have a disease or trait to determine if the frequency of alleles or genotypes of these patients differ from controls who do not have the disease or trait. The case subjects are patients who have been definitively diagnosed with the disease being investigated; the controls are known to be unaffected. If a difference in the frequency of an allele or genotype of the polymorphism being tested between the two groups is observed, it indicates that the genetic marker may increase or decrease risk of the disease or likelihood of the trait, or be in linkage disequilibrium with a genetic variant which does. Likewise, the analysis of a case-control association study makes use of a simple approximation in the odds ratio. However, while case-control association studies are relatively easy to design and implement, they are prone to bias and confounding. In order to minimise bias, care must be taken in the selection of both cases and controls, in establishing definitions of disease, risk factors and in ensuring there are no confounding associations between detection of disease and risk factor exposure. Confounding factors must be identified prior to the start of the study. Individual cases can be matched to controls where is it thought that other factors, aside from the genotype of interest, might contribute to the development of disease and confound the causal association under investigation (436). Cases and controls are commonly matched by age and sex, and care must be taken to avoid population stratification unduly influencing the result of the analysis (population stratification will be discussed in more detail in section 7.2). The single largest drawback to a case-control association study is that the results, by definition, only

reveal an association of the variable being investigated with the disease. If a genetic association is noted with a disease, it does not provide evidence of causation between the disease and the genetic variant (437).

If the limitations inherent to case-control study design are adequately addressed, then association studies are very useful when searching for genes which confer a relative risk of between 1.5 and 4.0. The sample size required in order to detect an effect of that magnitude is much smaller than would be required through linkage (438). As illustrated in Figure 7-1, assuming an allele frequency of 0.2 in the population being tested, you would require approximately 500 samples in order to detect an allele with a genotype relative risk of 2.0. Approximately 3500 would be required in order to capture an allele of the same frequency with a genotype relative risk of 2.0 via linkage. Thus, based on the composition and size of the patient cohort available, a case-control association study is clearly the optimal study design for experiments conducted in this thesis.

## 7.2 – Cohort Selection and Utilization

Throughout the experimental work presented in this thesis, there has been repeated use of a PsA cohort from Newfoundland, and a second PsA cohort from

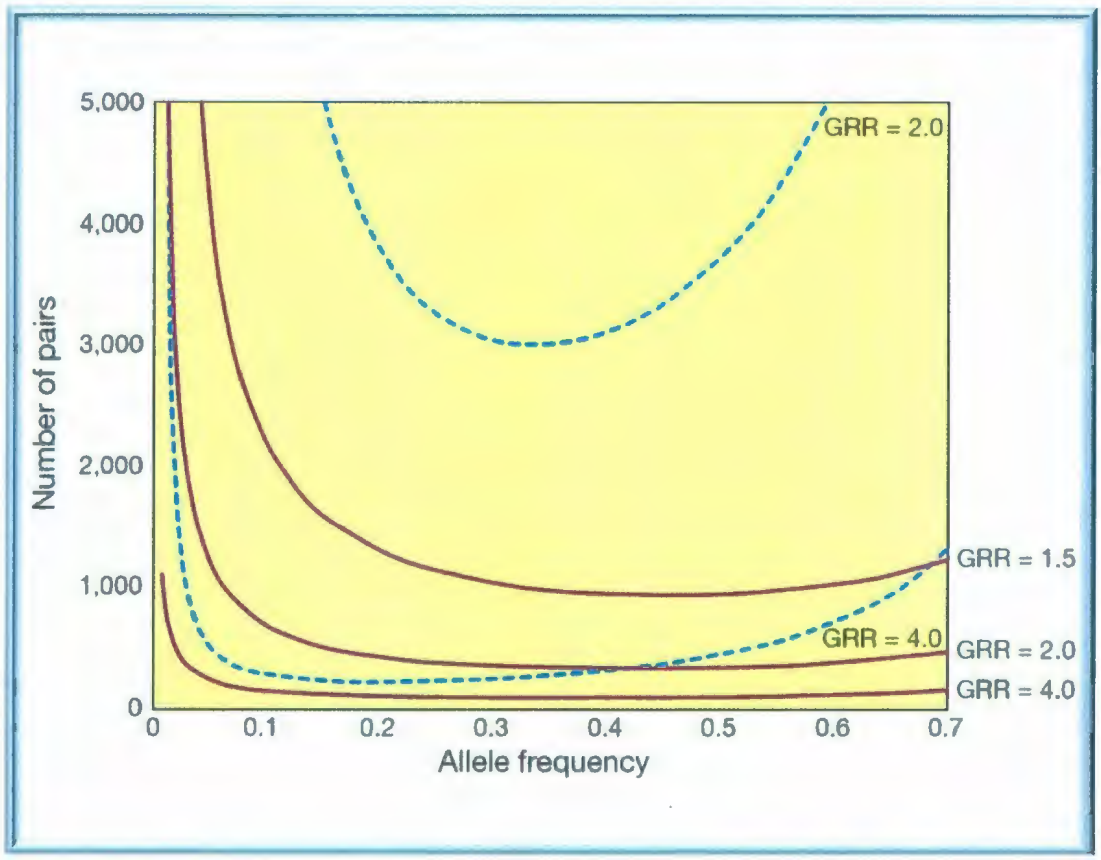


**Figure 7-1: Comparison of sample sizes required to detect a genetic effect via linkage (dashed lines) and association (solid lines).**
Adapted from Risch, 2000

Toronto. While combining both patient cohorts into one group for genotyping would have provided the studies presented here with a larger sample size and thus increased the statistical power of the findings, it was decided to analyze them separately in order to control for the possibility of population stratification.

Population stratification refers to differences in allele frequencies between cases and controls due to differences in ancestry rather than association of genes with disease. Stratification can be a problem for association studies, as an observed association could be due to the underlying structure of the population rather than a genuine disease associated locus. Conversely, a true association might not be found if the locus is less prevalent in the population where the case subjects are chosen. While it has been shown that the Newfoundland population does not represent a true population isolate or founder population, with levels of linkage disequilibrium comparable to that of an outbred European derived sample and the Afrikaner population of South Africa (304), the fact remains that Newfoundland was founded by a limited number of settlers and the population grew largely through internal expansion rather than in-migration (305). This combined with religion-influenced mating segregation in early Newfoundland history, and geographical isolation of communities could potentially lead to population stratification.

One proposed method of controlling for population stratification has been to genotype unlinked markers. When this method was tested on 44 unlinked markers in four case-control studies, stratification was detected in only one study, although the signal was no longer apparent after more stringent matching of

cases and controls based on the birthplaces of the individuals' grandparents. This has been interpreted as evidence that stratification may be less of a concern than originally anticipated (439). A larger study by Freedman *et al*, in Nature Genetics in 2004 analyzed unlinked SNPs in 11 different studies (440). None of the 11 studies showed statistically significant evidence of stratification after correcting for multiple hypotheses testing, however the authors did state that assessments based on a few dozen markers lack power to rule out moderate levels of stratification that could cause false positive associations. Other evidence, however, has shown that the effects of stratification can be largely overcome by carefully matching cases and controls according to self-reported ancestry and geographical origin (441). Therefore, based upon the cumulative evidence of these studies regarding the nature and likelihood of population stratification, and despite the potential loss of statistical power, the experiments detailed here were performed by using both PsA cohorts separately – the Newfoundland cohort was used for initial investigation, and the Toronto PsA cohort was used for replication.

## 7.3 - Marker Selection

At the time the experiments detailed in this thesis began in 2004, case-control association studies were usually carried out as hypothesis-testing research. Variants in a single gene or small number of genes with a known or putative role in a disease process were genotyped in patients and control

subjects usually from within one centre. As the sample sizes in these studies were typically small, the need for statistical correction was minimal. Results often overestimated the true genetic effect size as a consequence of the "winner's curse" phenomenon. Simply put, the genetic effect in an association study is biased upward, conditional on that study being the first to reach statistical significance and be published (442).

The first genome-wide association study (GWAS) was published in 2002, and used 65,761 SNPs in 94 cases (443). GWAS were thought to be especially useful in the study of complex disease, as it changed the focus from hypothesis-driven research to hypothesis-generating research. As the pathogenesis of many common diseases was not fully elucidated, it was hoped that finding genetic associations would yield clues towards disease process and progression. 2005 marked the first year that multiple GWAS were published (444,445), and with the introduction of large, highly accurate (0%–2% missing data; <0.5% errors) 1 million marker ``SNP chips`` from Affymetrix and Illumina in 2007-2008, the cost of performing a GWAS has dropped immensely (approximately $500 [U.S.] per subject; an approximate 2,000-fold reduction of cost per genotype in just under 10 years), (446) making them much more feasible. While performing a GWAS has become more feasible, the number of SNPs now being tested means that after corrections for multiple testing even GWAS using 2000-5000 samples are, in fact, underpowered to detect genetic effects of even modest sizes (447). Were the studies described in this thesis initiated today, they would undoubtedly contain a greater SNP density due to the lower cost of genotyping, and efforts

would be made to collaborate with other groups in order to initiate well powered GWAS of PsA. The studies presented here have attempted to fully utilize the technology available to us at the time they were conducted by performing association studies in accordance with the literature standards of the time, and by later using large numbers of SNPs to fine-map and haplotype regions associated with PsA (See chapters 4.2 and 6).

## 7.4 - Patient History, Lifestyle and Environmental Factors

A potentially large source of confounding error in the studies presented in this thesis is the lack individual patient medical histories, and their environmental exposures. There is considerable evidence that infection may play a role in the development of PsA. Acute episodes of guttate psoriasis have been associated with streptococcal infection (448), and one study indicated that the prevalence of positive streptococcal throat swabs in *HLA-Cw*0602* positive patients was twice the prevalence among *HLA-Cw*0602* negative patients, while no difference was observed among *HLA-Cw*0602* positive versus negative controls. In sub-Saharan Africa PsA is almost universally associated with HIV infection, even though prior to the HIV epidemic, seronegative spondyloarthropathies were considered uncommon in Africans (449,450). Genetic factors responsible for the immune response to infection, or the nature of the infectious agent may play a role in the development of PsA.

Trauma has long been associated with psoriasis (the "Koebner phenomenon" (107)), and several articles have reported on the triggering role of trauma in inducing arthritis in patients with psoriatic disease (451-453). As reviewed in McGonagle *et al*, 2009 (106) the recent recognition of a synovio-entheseal complex (SEC) may help resolve the relationship between synovitis and enthesitis in PsA, while also advocating a role for a localized Koebner phenomenon. The SEC model shows how enthesis fibrocartilage could derive lubrication and nourishment from adjacent synovium in a manner identical to the long appreciated interaction between articular cartilage and synovium. Hence, all sites of disease in PsA could potentially be linked to the common denominator of enthesitis. Several micro-anatomical studies have shown that there appears to be an 'enthesis organ' which is prone to microdamage at insertion sites and adjacent tissues, microscopic inflammatory and vascular changes both at and near the insertion itself and the immediately adjacent synovium. Given the known link with skin psoriasis and trauma, these findings of clinically unrecognized trauma at insertions sites are especially noteworthy. It is a distinct possibility that genes involved with the structure of tendon and ligament insertion points, or which control the marrow fluid that occupies the spaces between the bone and insertion points, or genes responsible for the response to such microtrauma may be quite important in the pathogenesis of PsA.

Smoking has also been linked to a large number of immunologically mediated diseases. In the western world, smoking is the most well established environmental risk factor for IBD, increasing the risk by approximately two-fold

(454), and at least one study has shown that the expression of certain genes is upregulated in the descending colon mucosa in smoking Crohn patients versus patients who have never smoked (455). Tobacco use has also been shown to be associated with the risk of development of psoriasis (456) and with poor long term outcome in AS patients (457,458). Direct gene-environment interactions have also been observed with respect to smoking and rheumatic disease: one study has reported a significant multiplicative interaction between the 1858 C>T SNP of the *PTPN22* gene and smoking for more than 10 pack-years (459), and a history of smoking has been shown to interact with the presence of *IL-13* polymorphisms to increase the risk of PsA (460).

Clearly, patient history and lifestyle play a role in the development of diseases and may modulate the course of autoimmune disease. Analysis of patient history along with genotypes could provide interesting data with respect to gene-environment interactions.

## 7.5 – DNA Source May Contribute to Genotype Results

All DNA samples used in the studies presented in this thesis were extracted from white blood cells of venous blood. This method is the most commonly used manner of obtaining DNA from patients who do not require invasive medical interventions. However, a growing body of evidence suggests that DNA obtained from sources other than those primarily involved in disease

processes (i.e., in PsA, from the sites of psoriatic skin lesions, or from synovium inflamed joints, or enthesis sites) may not be the most appropriate for analysis for genetic association.

One recent study established that there is tissue-specific splicing of DNA (461). The authors studied the genetic control of both exon-level and whole-transcript level variation in expression in two cell types (white blood cells, and cortical brain tissue from a set of control individuals), combined with parallel genome-wide genotyping of these samples. Using identical genome-wide screens in two primary tissue types allowed them to identify polymorphisms with clear effects on both overall expression and splicing, and to show that these effects are often tissue specific. A total of 23 high confidence associations with total expression and 80 with alternative splicing as reflected by expression levels of specific exons were identified. Fewer than 50% of the implicated SNPs showed effects in both tissue types, reflecting strong evidence for distinct genetic control of splicing and expression in differing tissue types. When the results were compared to reported associations of SNPs in genome wide scans of diseases, the authors noted several overlaps, including an over-representation of autoimmune diseases such as AS, Crohn, RA, and T1D. Most of these connections appeared to relate to alternative splicing as opposed to overall expression changes, suggesting that changes in splicing patterns may be more consequential for disease than those which affect only gene expression. Of specific interest to the work presented in this thesis, splicing effects of a SNP in the *PTPN22* gene were observed. The authors postulate that excess of

representation of autoimmune disease in these tissue-specific splicing variants may be due to the growing recognition of the importance of rare genetic variants in common disease (462,463). Specifically, the authors state that: "It is generally assumed that when a common SNP is associated with disease in a genome-wide study, that it, or some other common variant in LD with it, is responsible for the association. It is theoretically possible, however, that many of the associations observed are not due to single common variants, but rather due to a constellation of more rare disease-causing variants that happen to occur, by chance, more frequently along with one of the common alleles at given SNP as opposed to the other. In such a case, the signal of association credited to a common SNP is actually a synthetic association resulting from the contributions of multiple rare SNPs. In such cases a screen for a common SNP associated with an underlying biological effect (such as expression or splicing) is not likely to identify a causal site."

Further to differential control of splicing in various tissue types, another study has shown that there are significant differences in the SNPs observed in DNA from diseased tissue versus non-diseased tissue and blood (464). While this study was examining the genetic contribution to abdominal aortic aneurysm (a multifactorial, although non-autoimmune disease), the results are interesting and relevant to the critique of this thesis. The authors observed specific *BAK1* SNP alleles in both aneurysmic (31 cases) and healthy aortic tissue (5 cases) without seeing them in the matching blood samples. These same *BAK1* SNPs have been reported, although rarely (average frequency < 0.06%), in reference

*BAK1* DNA sequences. The potential that rare variants from reference sequences could be more frequent in diseased tissue presents an interesting challenge to the current methods of performing association studies or fine mapping of regions. At the current time, common variants (typically with a minor allele frequency greater than 5%) are chosen from the HapMap data for genotyping in order to capture other variants through LD, while rarer variants are typically not used. The authors of this study hypothesize that multiple variants of genes may pre-exist in "minority" forms within specific tissue in the non-diseased state, and may be selected for when intra- and/or extracellular conditions change, leading the tissues to be predisposed to an increased genetic susceptibility to disease. This tissue specific increased predisposition would not be identified in a routine genetic analysis of DNA from blood, and thus could easily lead to false-negative results for genes with plausible biological mechanisms in disease pathogenesis.

## 7.6 - Results Summary

This thesis has attempted to take a comprehensive genetic approach towards the study of PsA and have examined polymorphisms in genes involved in the several different pathogenic aspects of the disease. The research presented here has identified significant associations with pro-inflammatory genes in the MHC region (*TNF-α*, *MICA*) and established them as genuine associations via meta-analysis of previously published works. The work

presented here is among the first to examine genes involved in angiogenesis, observing associations with both *PPAR-γ* and *VEGF*. The association with *VEGF* is particularly noteworthy, as this gene also plays a role in inflammation. The results regarding T-cell activating *PTPN22* gene have proven controversial with respect to PsA; however, we have observed an association which has been replicated (228-230), adding to the evidence that this gene may play a role in several immunologically mediated diseases.

While not as exciting as positive associations, negative associations are important results as they exclude targets. This research is also among the first to examine genes involved in bone morphogenesis, observing no association between the *OPG, RANK,* and *RANKL* genes. We have also been able to narrow the possible factors for the overlap between PsA and conditions such as RA and Crohn disease by eliminating *NFKB1, NFKBIA, RELA, SLC22A4*, *SLC22A5, SLC9A3R1* and *RUNX1* as candidate genes for PsA. Likewise, the failure to replicate SNPs in the *IL-1* gene cluster – while confounding results observed in the Newfoundland cohort - serve to add to the information scope of the impact of high-priority candidate genes, and remind us that all results from a small, relatively homogenous population must be interpreted with caution due to the possibilities of population stratification and founder effects. Finally, we are the first research team to analyze epistatic interactions in PsA, finding an association between variants of *Il23-R, PTPN22,* and *PPARγ,* and the first to propose an algorithm capable of examining more than 6 epistatic interactions (421). The results obtained from the epistasis analysis are particularly interesting as they

illustrate once again that a complex disease is likely due to the interplay of several polymorphisms in different genes, each individually carrying only a small risk.

To summarize, the proper design and execution of association study has been demonstrated here, by the use of several study motifs: univariate and multivariate analysis, meta-analysis, small and large-scale haplotyping, and the use of computational power to address epistatic interactions. Through careful design and analysis, the data collected here represent a large contribution to the body of knowledge regarding the genetics of PsA.

## 7.7 Context of Results

A model has been proposed which ties together genetic and immunologic elements for the development of psoriasis and PsA (465). While the precise role of *HLA-Cw6* in psoriasis remains unknown, it has been postulated that it may be involved in antigen presentation to CD8+ T cells, whose migration into the epidermis appears to be required for the development of psoriatic lesions (466). During a flare of streptococcal infection, CD8+ T cells encounter strep antigens presented via HLA-Cw6 in the tonsils, where they proliferate and then migrate into epidermal tissue. This migration causes activation of macrophages and dendritic cells, which release TNF-α, which further facilitates the entry into epidermis, and thus begins activation of several TNF-α receptor mediated

pathways (via macrophages) which then signal to NF-κB in the nucleus. Genetic alterations in the tumour necrosis factor α interacting protein 3 (*TNFAIP3*) and TNFAIP3-interacting protein 1 (*TNIP1*) genes may play very important roles in antigen presenting cells and macrophages because of the central role of these genes in regulating NF-kB signalling (note that *TNFAIP3* and *TNIP1* have been noted to be associated with psoriasis via GWAS (241)). A subset of these CD8+ T cells will express Il-17 (known as Th-17 cells). As production of Il-17 is partially stimulated by Il-23 and Il-1, variations in the *Il-12B*, *Il23A*, *Il-23R* and/or *Il-1* gene cluster and associated subunits and receptors, genetic alterations in any of these genes could cause an upregulation in the number of Th17 cells present. These cytokines stimulate keratinocyte proliferation and up regulate keratinocyte innate immune defence mechanisms, including defensin, psoriasin, and other proteins that are highly expressed in psoriasis lesions, provoking epidermal hyperplasia.

While this does explain some of the genetic involvement in the immunology of psoriasis, clearly something more must occur in order for uncomplicated psoriasis to proceed into PsA. As virtually all of the cytokines described in the preceding paragraph also play a role in synovial inflammation, bone remodelling, enthesitis, and the initiation of angiogenesis (as described in section 1.6), further genetic variation in any of the described genes in these pathways could lead to the development of arthritis accompanying psoriasis. The fact that PsA typically develops 7-10 years after the onset of psoriasis gives adequate time following a triggering event for the dysregulation of skin histopathology to migrate further into bone and joint areas to cause PsA if

predisposing genetic variation is present. The results of our epistasis algorithm, showing the interaction of variants in *Il23-R, PTPN22,* and *PPARγ* serve to reinforce this hypothesis.

## 7.8 – Possible Future Directions

Despite the successes, it is important to keep these results in context as there are criticisms which can be fairly attributed to these studies. PsA is a heterogeneous disease with several endophenotypes. Stratifying arthritic disease according to endophenotype has previously been shown to be a successful method in order to identify genetic association when none was evident prior to stratifying patient groups (467). The problem with this is that in performing such stratification, we reduce sample size and therefore power to detect an association. Given that PsA has a population prevalence of 0.25%, and that the population of Newfoundland and Labrador is approximately 507 900, there is approximately a maximum of 1270 PsA patients in Newfoundland and Labrador. Given the extreme population spread on the island of Newfoundland, and the division of health care services into regional health boards, we are likely now approaching the maximum number of PsA patients available. Further reducing the sample size by stratifying patients would likely jeopardize the opportunity to observe a gene effect if one is present. Also, population stratification in the structure of the Newfoundland population is an important characteristic to consider. As most residents of Newfoundland are largely descended from

immigrants from small areas of southwest England and southeast Ireland, there are likely to be founder effects in the genetic structure of the population. This is quite evident as there is an over representation of monogenic diseases in our population – approximately 10 fold higher than the other Atlantic Provinces (305). This evident founder effect could also serve to bias our results in both positive and negative fashions: it is possible for instance that our observed positive associations could be the result of a gene variant that is simply present in our population at a higher frequency than other admixed Caucasian populations due to the founder effect. Likewise, it is possible that our inability to replicate candidate gene variants observed in other populations could be due to an under representation of that variant in the founding population.

PsA does present unique challenges in the study of complex disease when compared to others, as it represents a "disease-within-a-disease". As described previously in approximately 70% of PsA cases psoriasis precedes the onset of arthritis by between 7 – 10 years (25), and only about 30% of patients with psoriasis develop PsA (8). Distinguishing between genetic variants responsible for PsA and those responsible for uncomplicated psoriasis is a large challenge for well designed studies of PsA; however the time of onset of PsA from psoriasis does provide investigators with a potential mechanism to investigate genetic differences between the two. By performing a case control study using psoriasis patients who have had established disease for longer than 10 years as a control group, an investigator will have ensured that the control group is unlikely to develop PsA, as they have surpassed the typical time frame

for development of PsA. Thus, any differences in allele frequencies between the PsA cohort and psoriasis control cohort may represent genuine PsA markers. For those markers which appear to be associated with both conditions, a comparison of the relative risk for each group could potentially reveal a real PsA association: as PsA represents a "disease-within-a-disease", an associated marker has the potential to appear in both PsA and psoriasis groups. If the calculated relative risk is higher for the PsA cohort as opposed to the psoriasis cohort, then that marker is likely to represent a PsA risk allele. The stratification of PsA patients by endophenotype can serve to further strengthen this finding by examining the marker in an AS cohort as well. If the marker is found to be associated with the spondylitis sub-form of PsA, and it is found to be associated with AS, then it is more likely to be involved with arthritic disease as opposed to psoriatic skin disease.

A further manner in which to identify true PsA genes is through the use of expression studies. There are medications which can have different effects on psoriasis and PsA. The drug efalizumab (also known as Raptiva, which has recently been removed from the market) effectively treats psoriasis patients and the psoriasis of PsA patients; however it does not affect the arthritis portion of the disease, and may in fact worsen the course of arthritis (468,469). Examining the gene expression levels of PsA patients before and after treatment with efalizumab would likely provide more evidence regarding genes involved in PsA rather than uncomplicated psoriasis, as you would expect the expression levels of psoriasis-related genes to drop following treatment. Genes that still remained

over or under expressed after treatment when compared to healthy controls would likely be involved in the arthritis portion of PsA.

More recent work has identified another source of genetic influence on psoriasis, as micro RNAs (miRNAs) have been implicated in the pathogenesis of psoriasis (470). MicroRNAs (miRNAs) are an abundant class of short (~22 nucleotides), non-protein-coding RNAs that regulate the expression of protein-coding genes at the posttranscriptional level. Specifically, the authors of this study identified the first miRNA that is entirely restricted to skin epithelium and showed that it is over expressed in psoriasis. One of the targets of this miRNA for post-transcriptional suppression is a negative regulator of the STAT3 pathway, which is activated by inflammatory cytokines (e.g. Interleukin-6, interferon-γ) and functions in the regulation of both innate and adaptive immunity. Constitutive activation of this pathway has been reported to lead to a psoriasis-like phenotype in transgenic mice, stressing its relevance in psoriasis (471). Their results suggest that the increased expression of this miRNA leads sustained activation of the STAT3 signalling pathway, and thus may contribute to increased or prolonged skin inflammation in response to T cell-derived cytokines, due to a seemingly aberrant negative feedback in cytokine signalling in keratinocytes. Interestingly for the pathogenesis of both psoriasis and PsA, two other miRNAs (one which was found to be significantly over-expressed, and one which was significantly under-expressed) were direct mediators of the TNF-α pathway. Clearly, future genetic studies of PsA should examine these emerging genetic regulators.

Copy-number variation (CNV) is also another emerging area of genetic study that is worthy of consideration in the investigation of PsA, as data has already established that a deletion comprising the genes *LCE3B* and *LCE3C* is significantly associated with risk of psoriasis (472). Likewise, a higher copy number of the β-defensin gene cluster found on chromosome 8 has been demonstrated to be a susceptibility factor for psoriasis (473). While no specific CNVs have been found for PsA, others have been found to be associated with susceptibility to autoimmune and inflammatory disorders (474). As the extent of CNVs in the human genome has only recently begun to be fully appreciated (475,476), investigating this form of genetic variation could lead to new insights regarding the genetic contribution to PsA.

Regardless of the method used to investigate the genetics of PsA, the difficulties in study design and analysis can be overcome. As described previously, the choice of adequate control groups is crucial. The ideal genetic study of PsA (whether through SNPs, CNVs, miRNAs, or direct sequencing) would consist of large (the ideal sample size being over 1000), well characterized and stratified PsA patient groups (according to pattern of arthritis, and also according to whether the cases were sporadic or had a family history), but would also contain multiple control groups. In addition to normal, healthy controls, control groups should consist of uncomplicated psoriasis patients, AS patients, and in addition to this every effort should be made by researchers to collect and characterized as many PsA patients as possible who develop arthritis prior to psoriasis - what is known as "psoriatic arthritis *sine* psoriasis" (477,478). The

inclusion of sufficient numbers of these proposed control cohorts would allow investigators to definitively distinguish between variations associated specifically with PsA as opposed to psoriasis. Comparison of DNA from disease sites as well as that from white blood cells would also be beneficial. In addition to identifying rare variants involved in common disease, examining tissue from these multiple groups of patients with autoimmune diseases would help to characterize the exact disease processes involved across multiple autoimmune diseases, and further help to elucidate the common shared pathways in these types of diseases (300).

Finally, the possibility also exists that the genetic nature of PsA is more complex than we currently understand and thus, better targets than the ones we have chosen to investigate exist. There has, at the time of this writing, been only a single genome wide association study performed specifically in PsA. As we approach the post-genomic era and more and different markers are available for analysis such as the locations of Copy Number Variants and non-coding RNA silencers, the genetic analysis of complex and uncommon diseases such as PsA will become easier, especially as computational problems towards solving epistatic interactions between all of these variants are overcome, and the networks of interactions of all of these genetic elements are more clearly established.

As a final summary of the work in this thesis, Figure 7-2a describes all candidate genes which to date have been analysed in PsA. Figure 7-2b indicates

those which have reported positive associations, and figure 7-2c shows which have been replicated by subsequent reports.

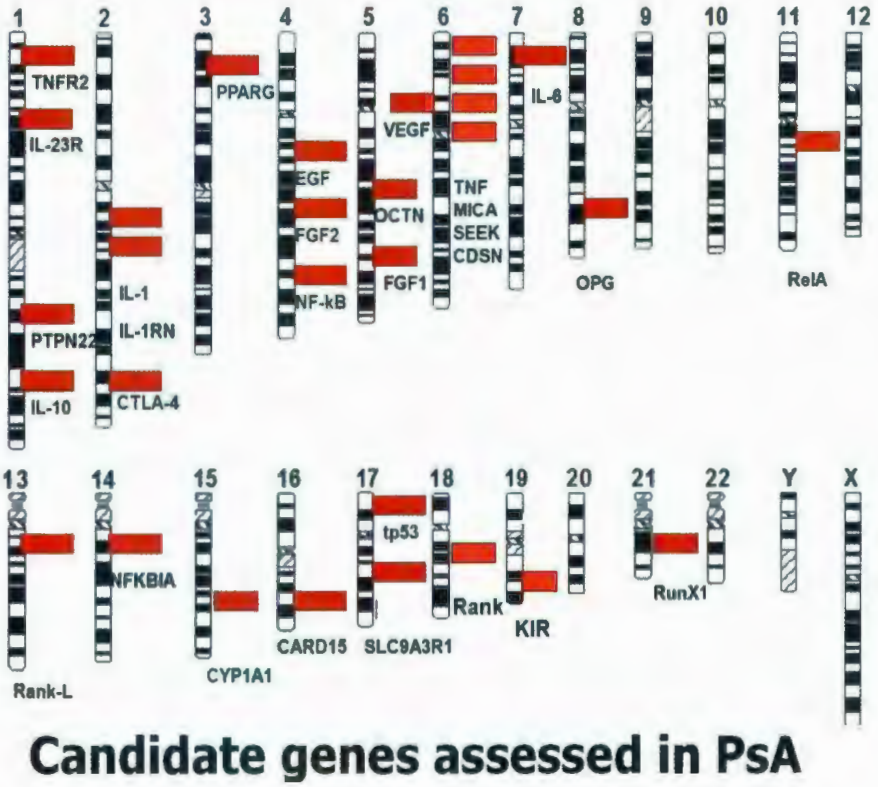


Figure 7-2a: Summary of all candidate genes for PsA assessed in the literature up to September 2009.

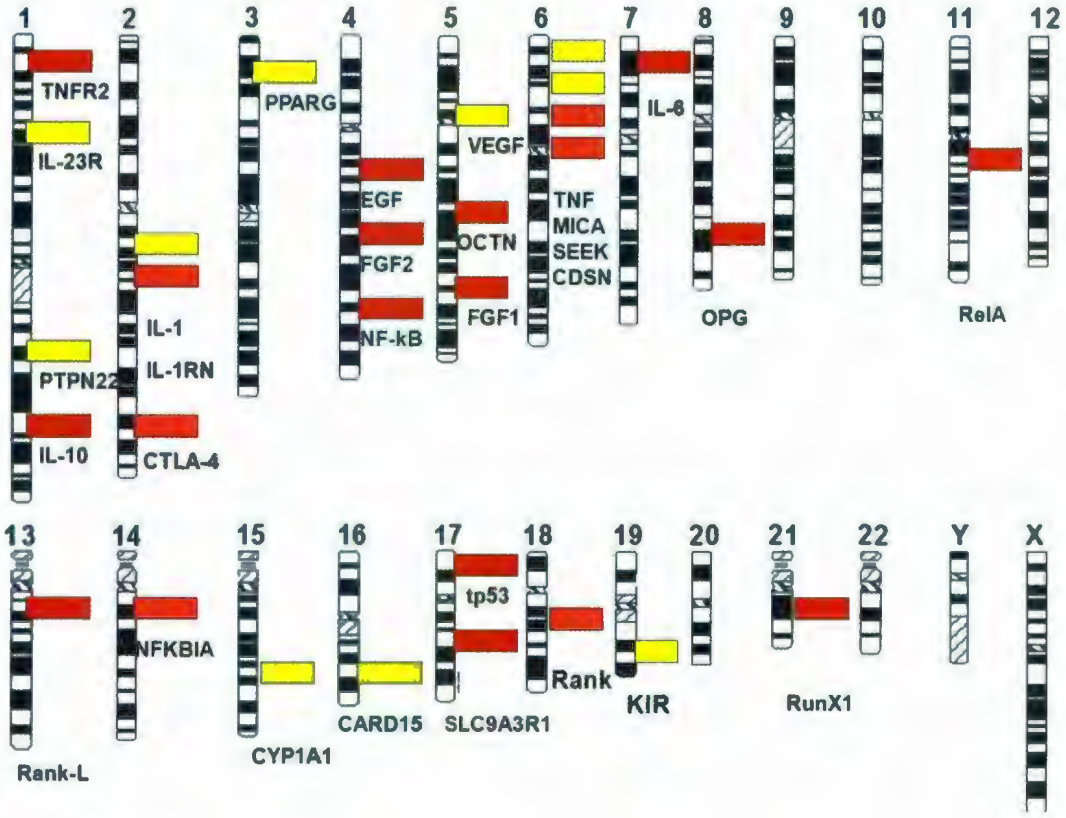


Figure 7-2b: Genes in yellow indicate positive reports of association with PsA, current as of September, 2009.

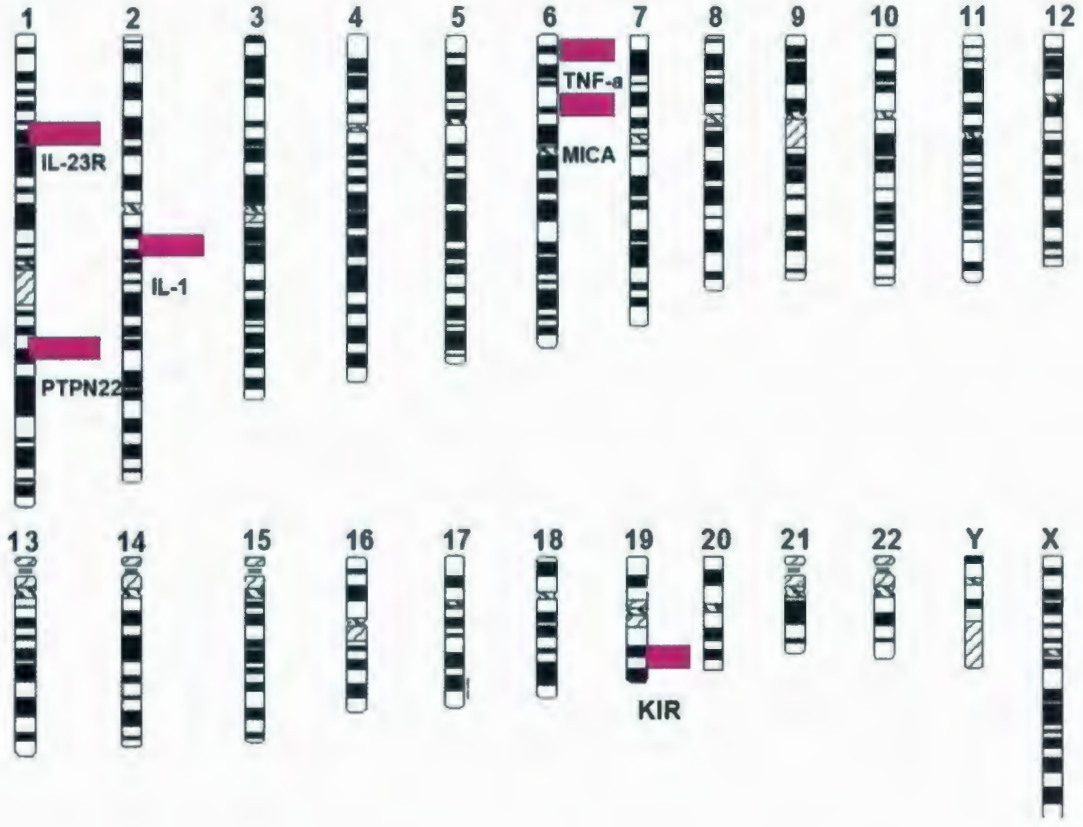


Figure 7-2c: Genes shown here are the only candidate genes with replicated associations with PsA reported in the literature – current as of September 2009.

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# Appendix 1

## Epistasis algorithm and validation

**Introductory Note**

The algorithm which follows was originally written by Mr. Mohammed Uddin as part of his Master's dissertation (2008). The algorithm is reprinted here with his kind permission. Mr. Uddin remains the sole copyright holder of this algorithm, and it is entirely his intellectual property.

**Co-operative Coevolutionary Algorithm (CCA)**

There is no recognized single best method to detect gene-gene interactions comprehensively. Moreover, it is not feasible to perform exhaustive search on non-trivial amounts of single nucleotide polymorphism (SNP) data to detect gene-gene interaction. A case-control cohort consists of $m$ case and $m'$ control individuals for a panel of $n$ SNPs; hence, the case and control matrices $M$ and $M'$ consist of $2m$ and $2m'$ haplotype-rows, respectively, and $n$ SNP-columns. In each column, let 1 and 0 denote the major and minor alleles, respectively, of the SNP associated with that column and "—" denote a missing value. The size of the search space of all haplotypes with contiguous and non-contiguous SNPs in $M$ and $M'$ is at least $2^n$ (each row in $M$ and $M'$ is identical) and at most $(2m \times 2m')2^n$ (each row in $M$ and $M'$ is different). Given the size of this search space, any usable algorithm for large datasets must sample this search space in a quick yet approximately comprehensive manner. A genetic algorithm (which

approximates optimal solutions by iteratively evolving a population of initially random solutions) is one such type of algorithm. We use a variant of standard genetic algorithms called cooperative co-evolutionary genetic algorithms (CCA), which were defined by Potter et al. (479). The main distinction between a standard genetic algorithm and a CCA is that the latter simultaneously evolves multiple subpopulations, where each subpopulation evolves a portion of a solution for the given problem $\Pi$ and members of these subpopulations are combined to create complete solutions for $\Pi$. Since each subpopulation's search space is smaller than the complete solution search space, CCA may find better solutions faster than standard genetic algorithms (480), and seems in practice to give better performance for various complex problems (481). Pseudocode for our CCA for detecting allelic interactions of genes from case-control haplotype data is shown in Figure 1. In this algorithm, each member $x$ of subpopulation $S_k$ corresponds to a haplotype-fragment over a certain set of contiguous SNPs. This haplotype-fragment is encoded as a vector of subpopulation-specific length $l_{(S_k)}$ such that there is a 1, 0, or * at each position, where 1 and 0 represent the major and minor alleles of the SNP associated with that position and * means that this SNP is not included in the haplotype-fragment. A complete solution is a concatenation of subpopulation members forming a vector of length $n$ such that here is a 1, 0, or * at each position. Details of the central aspects of and operations in this algorithm are given in the following subsections.

1. *gen = 0*

2. for each subpopulation $S_k$ ,
3. randomly generate initial subpopulation members, i.e., $S_{\{k,0\}}$
4. while ( $gen < gen_{\max}$ )
5. for each subpopulation $S_{\{k,gen\}}$
6. select parent member(s) from $S_{\{k,gen\}}$ and apply genetic operators
7. perform best member collaboration to form a complete solution $C_{x,i}$
8. for each member *x* of $S_{\{k,gen\}}$
9. evaluate fitness of *x*
10. if (fitness of *x* > *t*)
11. compute Haplotype Risk Ratio (HRR) for $C_{x,i}$ and perform Permutation Test
12. apply Niching
13. perform Selection
14. *gen = gen + 1*

end.

**Genetic Operators**

We use two genetic operators in our CCA. The first operator (one-point crossover) is applied to a pair of parents in a subpopulation to produce an offspring. A random point *r* is drawn from the range 1 to *l*, where *l* is length of the members in that subpopulation. The offspring is produced by copying positions 0 to *r-1* from the first parent and positions *r* to *l* from the second parent. The second operator (mutation) is applied to a single parent to produce an offspring, and is an adaptation of the one-point bit-flip mutation operator. A position in the parent's vector is chosen randomly and that position is flipped by the following rules:

- if there is a 1, the bit is flipped to 0
- if there is a 0, the bit is flipped to *
- if there is a *, the bit is flipped to 1.

We have used random selection to select parents for genetic operator application in line 6 of the algorithm. The proposed CCA is steady-state, such that parents

and offspring compete with each other to remain in $S_k$ after the offspring are created. In particular, the fittest pair of the two parents and the offspring (in the case of crossover) and the fittest of the parent and the offspring (in the case of mutation) is selected and kept for subsequent processing.

**Collaboration**

Collaboration is a mechanism where a member from each subpopulation is picked to form a complete solution. We use the best member collaboration method proposed by De Jong et al. (480). In best member collaboration, the fittest member of each subpopulation is chosen as that subpopulation's representative. Each member *x* of a subpopulation is then combined with the provided representatives of other subpopulations to form a complete solution. The fitness of this solution becomes the fitness of *x* and is not shared with representatives of other subpopulations that participated in the collaboration. In our CCA, this process is first done relative to $S_1$, followed by $S_2$, and continuing to the other subpopulations.

**Complete-Solution Fitness Evaluation**

Our fitness function for complete solutions is based on the MDR approach proposed by Ritchie et al. for assessing the significance of haplotypes in case-

control SNP data (482). This approach computes case-control frequency differences for each multilocus genotype and if a difference exceeds a specified threshold, that haplotype is considered significant. Similarly, we define the fitness of a complete solution haplotype $C_{x,i}$ in terms of case-control frequency differences as follows:

$$f(C_{x,i}) = \left| fr(M, C_{x,i}) - fr(M', C_{x,i}) \right| \quad (2)$$

This in turn relies on estimates of haplotype frequencies specific to a case or control matrix *M*, which are computed as

$$fr(M, C_{x,i}) = \frac{\sum_{j=1}^{2m} \prod_{y=1}^{n} F(C_{x,i}, j, y)}{2m} \quad (3)$$

where,

$$F(C_{x,i}, j, y) = \begin{cases} 1, & if \quad M[j,y] = C_{x,i}[y] \quad and \quad C_{x,i}[y] \neq * \\ Pval(M, j, C_{x,i}[y]], & if \quad M[j,y] = - \quad and \quad C_{x,i}[y] \neq * \\ 0, & if \quad M[j,y] \neq C_{x,i}[y] \quad and \quad C_{x,i}[y] \neq * \end{cases} \quad (4)$$

Equation 3 computes the frequencies by scanning the Cx,i and ignores the computation for the positions $C_{x,i}[y] = *$, where $y \leq n$. Hence, this computation allows the algorithm to compute frequency for haplotypes with non-adjacent SNPs from matrix *M*. Frequency is computed by scanning each row in matrix *M*, one row at a time, and matching the content of each position with the content of $C_{x,i}$ at the corresponding position as specified in Equation 4. Function $Pval(M, j, C_{x,i}[y])$ is invoked when there is missing data in the matrix *M*. The

easiest and most commonly-used approach is to simply ignore such positions, i.e., $Pval(M, j, C_{x,j}[y]) = 0$; however, our algorithm uses a novel and much more intelligent approach that is described in a later section.

**Niching**

Maintaining the diversity of members in a subpopulation helps prevent the members of that subpopulation from prematurely converging to a local optimum as that subpopulation evolves. Niching is a method that maintains diversity and prohibits different members of a subpopulation from crowding into the same area of the solution search space (483). Our algorithm implements niching using the popular fitness sharing technique. This technique computes, for each subpopulation member *x*, the Hamming distance between *x* and all other members of a subpopulation and applies a fitness-reduction penalty to all other members that are close to *x*. As this penalty reduces the probability of a member being selected for the next generation, a high penalty rate can prematurely remove a useful member from the subpopulation and hence misdirect the evolutionary search. Therefore, we use a relatively moderate penalty rate of 15%.

**Measures of Haplotype Significance**

Several measures of haplotype frequency difference significance are computed for the purpose of assessing the quality of derived haplotypes but do not affect the haplotypes derived by the CCA, i.e., these measures are not part of fitness evaluation or subpopulation evolution. After haplotype frequency estimation, the Haplotype Risk Ratio (HRR) is computed for each $C_{x,i}$, and Pearson's $\chi^2$ test (1 d.f.) issued to quantify the significance level of each HRR value. No test for multiple corrections exists relative to the large number of tests performed by the CCA in each generation. Hence, we have applied the standard permutation test to reduce type 1 error (484). AS the permutation test is computationally expensive, it is only applied when the HRR $\geq$ 1.5 and the $\chi^2$ significance level is $10^{-3}$.

**Estimating Haplotype Frequencies for Missing Data**

Missing data in haplotypes arises primarily in two ways - when errors occur in genotyping and when the phasing algorithm is unable to resolve certain genotypes into haplotypes. To handle such data when computing haplotype frequencies, we use the non-missing alleles and their relative LD information to impute the frequencies of the missing alleles.

**Algorithm**

Let *L* denote the LD matrix, an $n \times n$ matrix that stores all pairwise $r^2$ values of the n SNPs from matrix *M*, where $r^2$ measure defines the correlation of alleles in the SNP pair, such that $r^2 = 1$ is known as complete LD and if the $r^2$ value between a pair of SNPs is greater than 1/3, the two SNPs are considered to be linked (414). Moreover, though LD decays as the distance between SNPs increases, extensive research has shown that an SNP can be linked with another SNP that is up to 100kb distant (414,485). Thus, the algorithm will consider a pair of SNPs as linked if their physical distance on the chromosome is within 100kb and their $r^2$ value is greater than 1/3. When *M[j, y]* contains a missing value "—", the function $Pval(M, j, C_{x,j}[y])$ finds a set of SNPs *Z* such that each $z \in Z$ is strongly linked with the *yth* SNP of matrix *M*, and uses this information to estimate the frequency of the alleles that can occur in $C_{x,j}$ in the position corresponding to the missing value *M[j, y]* as follows:

$$Pval(M, j, C_{x,j}[y]) = \frac{\sum_{z \in Z} fr(M, C_{x,j}[y], z)}{|Z|} \quad (5)$$

Following the guidelines described above, *Z* is constructed such that each *z* in *Z* is within 100kb with the *yth* SNP of matrix *M* and the $r^2$ value between *z* and *y* is > 1/3. It is possible to have an empty set *Z* for a set of loosely linked SNPs where all pairwise LD values in *L* are < 1/3; in this case, the set *Z* consists of all SNPs within 100kb of the *yth* SNP of matrix *M*. The function $fr(M, C_{x,j}[y], z)$ in Equation 5 gives the average frequency of the allele $C_{x,j}[y]$ from matrix *M*. Essentially, this

function computes the average frequency of allele $C_{x,i}[y]$ from the SNPs that are in set *Z*, which gives the approximate frequency with which allele $C_{x,i}[y]$ can occur in a missing position of the matrix *M*.

**Comparison of New Algorithm with Existing Approaches**

To compare haplotype estimation in the presence of missing data, we compared haplotype frequencies within haplotype blocks computed by our CCA with those computed by the popular analysis package Haploview (387) on 5 simulated datasets. To ensure realistic allele frequencies and SNP dependencies, we created our simulated datasets by mutating a real dataset cf. creation of simulated datasets *ab initio* from random allele sequences. Our source dataset consisted of 40 SNPs which span a 205 kb region on chromosome 2 genotyped from 401 ethnically matched individuals in Alberta population (data not shown). The haplotypes for this dataset were inferred for each individual using PHASE (348) and then used to create 5 progressively worse simulated missing-data haplotype datasets by randomly implanting 3%, 5%, 10%, 15%, and 20% missing data, respectively, in the PHASE-produced haplotype dataset. Haplotype blocks in strong LD and their associated haplotypes frequencies were then obtained for each simulated dataset using Haploview. Recall that Haploview uses an EM-algorithm to impute missing values during the computation of frequencies for haplotypes

with missing data. For each haplotype block, we compared the frequencies obtained by our CCA and the EM-algorithm using the mean square error (MSE) measure given below,

$$\text{MSE} = \frac{1}{H} \times \sum_{i}^{H} (h_i^{EM} - h_i^{CCA})^2$$

where H is the number of haplotypes in a block that are in strong LD, $h_i^{EM}$ is the frequency of $i^{th}$ haplotype in that block obtained by the EM-algorithm, and $h_i^{CCA}$ is the frequency of the $i^{th}$ haplotype obtained by the proposed method. Note that MSE captures the overall differences between the haplotype differences computed by the two methods.

**Experiment Setup**

The optimal values for parameters to run a genetic algorithm are still unknown but parameters of the CCA were set to maintain a balance so that the algorithm's evolution is not disruptive and does not demonstrate random search behavior. The crossover rate was set to 100% as one-point crossover is not as disruptive as uniform crossover (486). To exploit the fitness landscape with adequate chromosomal swapping, this rate of crossover is favourable. The mutation rate was set to 5%. 100 CCA runs were carried out for each four datasets. The AS and Schizophrenia datasets were separated into two subpopulations to evolve independently. Each subpopulation of the AS and Schizophrenia datasets consists of 10 and 5 SNPs, respectively. The PsA

dataset was separated into three subpopulations; each containing 10 SNPs. The member of each subpopulation in the AS dataset was set to 250 and each run evolves for 1000 generations. The subpopulations in the Schizophrenia dataset consists with 25 members due to it small number of SNPs. The strategy for the PsA cohort was to evolve large number of generations with small size subpopulation. Hence, each subpopulation with 100 members evolved for 1500 generations in each CCA run.

## Results

### Performance of Missing-Data Handling Algorithm

As expected, the MSE showed a positive correlation with error rate, such that error increased as the amount of missing data increased. However, below 15% missing data, there was no apparent difference observed between the haplotype frequencies obtained by our new method and the EM-algorithm in Haploview (Figure 2). In particular, for 3-15% missing data, the MSE varied between $4.12\times 10^{-6}$ and $8.79\times 10^{-3}$ for haplotype frequencies in a block, and at 20% missing data, the MSE varied between $2.36\times 10^{-3}$ to $1.61\times 10^{-2}$. This validates our haplotype frequency estimation method, in that its results did not deviate significantly from those produced by EM-algorithm estimation up to 15% missing data. Our proposed CCA is scalable to large data sets and can handle up to 15% missing/ambiguous data.

**Validation**

The cohorts for Ankylosing Spondylitis were taken from Maksymowych et al. (487). We have retained only the Newfoundland (NF) (112 AS cases, and 150 controls.) and Alberta (AL) (200 cases and 200 controls) cohorts for our analysis and excluded the Toronto cohort due to the possibility of population stratification. The ethnic background of the Newfoundland and Alberta population cohorts have been stable with little in-migration for several generations which is likely to make them much more homogenous than that of the Toronto cohort (305). The genotype data for both populations includes a set of 20 SNPs located on chromosome 2q14 which includes interleukin 1 alpha (*IL1A*; GeneID 3552), interleukin 1 beta (*IL1B*; GeneID 3553), interleukin 1 family member 7 (*IL1F7*; GeneID 27178), interleukin 1 family member 6 (*IL1F6*; GeneID 27179), interleukin 1 family member 8 (*IL1F8*; GeneID 27177), interleukin 1 family member 10 (*IL1F10*; GeneID 84639) and interleukin 1 receptor antagonist (*IL1RN*; GeneID 3557) in a 360 kb region. The Schizophrenia cohort was taken from Fukasawa et al. (488) in which 10 SNPs were genotyped from *Netrin G1* (*NTNG1*; GeneID 22854) gene located on chromosome 1p13.3. The genotyping was done on 180 cases with confirmed Schizophrenia and an equal number of ethnically matched controls from the Japanese population.

**Ankylosing Spondylitis**

After 100 runs of the CCA in our Alberta AS cohort, 53 haplotypes showed significance after 10000 permutations with $p < 5.0 \times 10^{-4}$ and HRR $\geq$ 1.5. The significant haplotypes consisted of major alleles from SNPs rs3783550, rs3783543, rs3783526, rs1143630, rs3917354, rs1143627, rs2723187, and rs3811058 located in genes *IL1A*, *IL1B*, *IL1F7* and *IL1F10*. The interaction captured by the CCA can be defined entirely by rs3811058 in the *IL1F10* gene because the major allele of this SNP is included in all haplotypes containing markers from *IL1A*, *IL1B,* and *IL1F7*. The most significant interaction observed was with the haplotype TT obtained from *IL1B* and *IL1F10* genes with a haplotype risk ratio (HRR) of 2.19. (95% CI 1.63 – 2.91). In our Newfoundland cohort, only two haplotypes were detected with smaller frequency differences between case and controls; AGCCTG ($p < 2.0 \times 10^{-4}$, HRR = 2.125(95% CI 1.445-3.142)) and AGCCG ($p < 1.0 \times 10^{-4}$, HRR = 2.144(95% CI 1.474-3.201)) and both haplotypes includes SNPs from genes *IL1A*, *IL1B* and *IL1RN* (Fig. 5). These results are particularly interesting as we have previously reported a univariate analysis of this data set and observed no significance (347), however, using our model in the absence of single locus association or haplotype analysis of sequential SNPs we observed three genes interacting with in two significant haplotypes.

**Schizophrenia**

Two analyses have been done in the *Netrin G1* gene region that is located on chromosome 1. Fukasawa *et al.* (488) conducted a case-control cohort analysis from the Japanese population that included genotypes for 10 SNPs in this *Netrin G1* region. The authors performed single window, 2-window and 3-window analyses to evaluate SNPs and their underlying haplotypes for possible susceptibility to schizophrenia. In their single window analysis, they have found significant association ($p < 0.05$) in SNP rs1373336. The 2-window and 3-window analyses showed significant association with haplotypes from SNPs rs894904, rs2218404, rs1373336, and rs1444042. The authors concluded that rs1373336 was the most significant SNP which has been detected by the three different analyses. In another independent study (489), the authors performed a family based analysis on a broader chromosomal region that also includes the 10 SNPs in the *Netrin G1* gene region. The authors reported strong association of SNPs rs4307594, rs3924253, rs1373336, and rs96501 in their single window results. In their 3-window analysis, the SNPs rs4307594, rs3924253, rs4132604, rs2218404, rs1373336, and rs1444042 showed susceptibility to Schizophrenia. The results in both studies detected the susceptibility of rs1373336 to Schizophrenia. In our results, we have found 8 haplotype patterns consisting of SNPs rs4481881, rs4307594, rs3924253, rs4132604, rs1373336, rs1444042, and rs96501 (see Figure). Our results also show that rs1373336 is the most significant SNP because all the haplotype patterns detected by the CCGA from the Schizophrenia cohort contain the major allele from SNP rs1373336. The

haplotype patterns that were captured by the CCGA contain major alleles in all SNPs except rs4132604, where the rare allele was included in the haplotype patterns. The most significant haplotype captured by our algorithm is ACT from SNP rs3924253, rs1373336, and rs96501, such that the HRR is 2.34 with $p < 0.0001$.

**Figure A-1: The mean squared error (MSE) measure for haplotype frequencies in 5 datasets in the presence of missing data.**

The plot illustrates the influence of missing genotypes on haplotype frequency estimation. Each line defines the error rate for a dataset, where the x-axis contains the number of haplotype block for that dataset and the y-axis is the corresponding MSE.

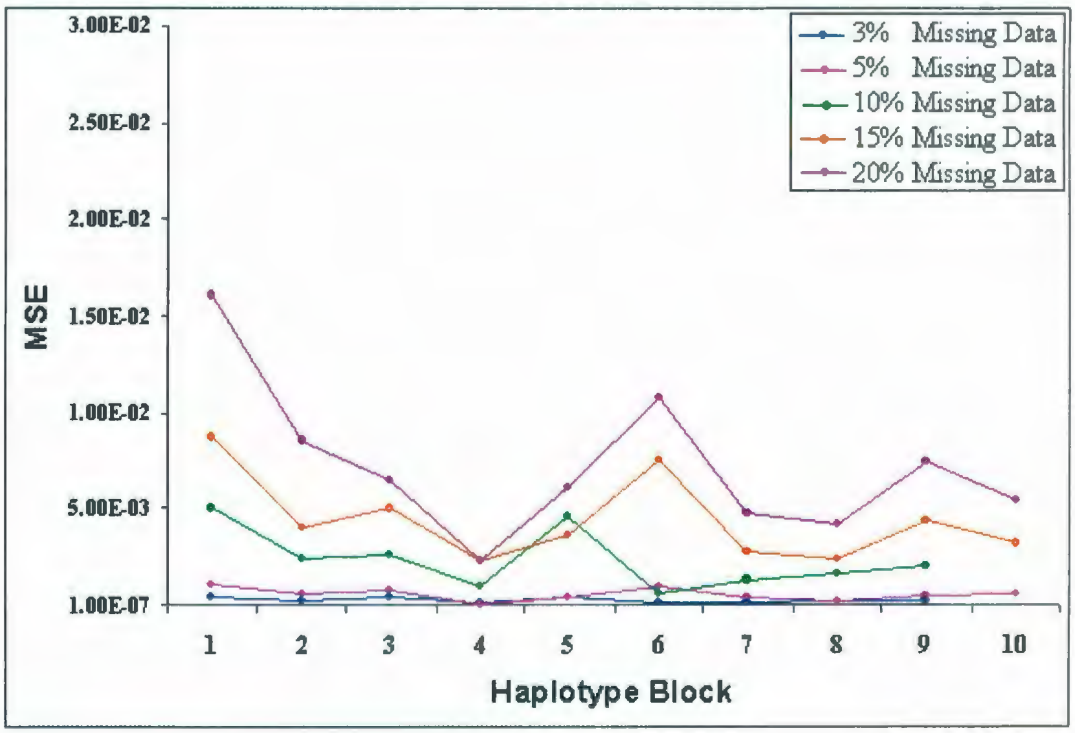
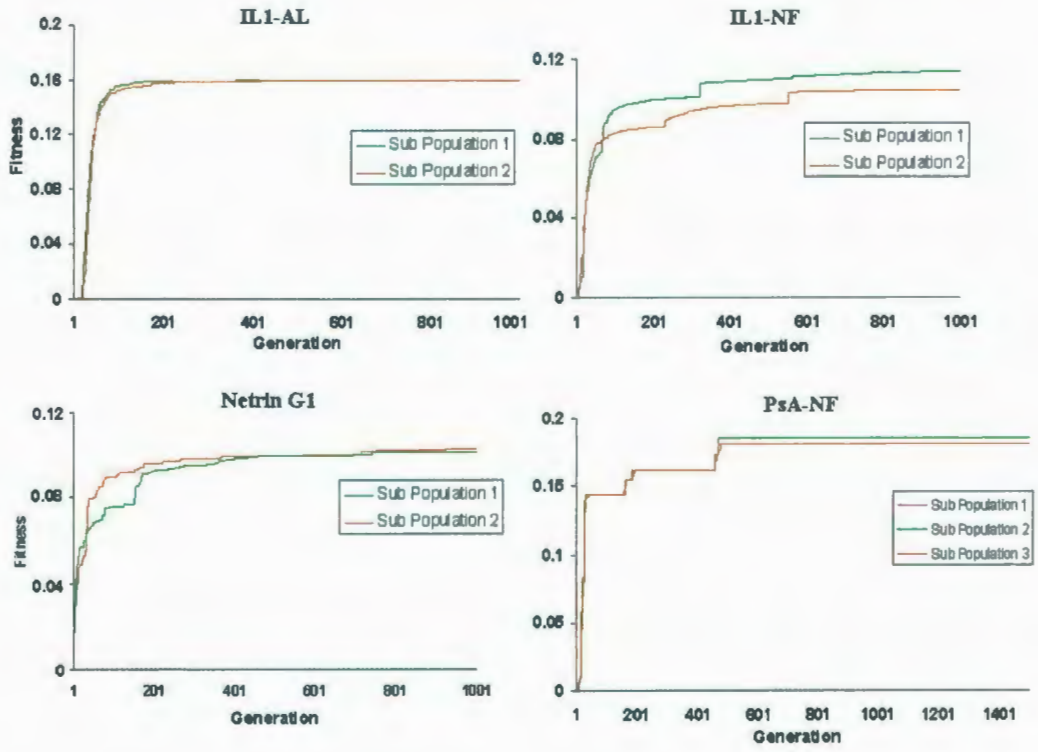


**Figure A-2: Evolution of each subpopulation in a typical CCA run.**

The subpopulation in each dataset shows similar behaviour of fitness progression in the initial generations and convergence after certain generations of evolution. The average fitness for each subpopulation was plotted in the y-axis relative to a single CCA run.



**FigureA-3: Fitness for 100 CCA run plotted for the IL1-AL, IL1-NF, Netrin G1, and PsA-NF dataset.**

The solid line represents the average maximum fitness in a CCA run and the dotted line is the average fitness for each CCA run.

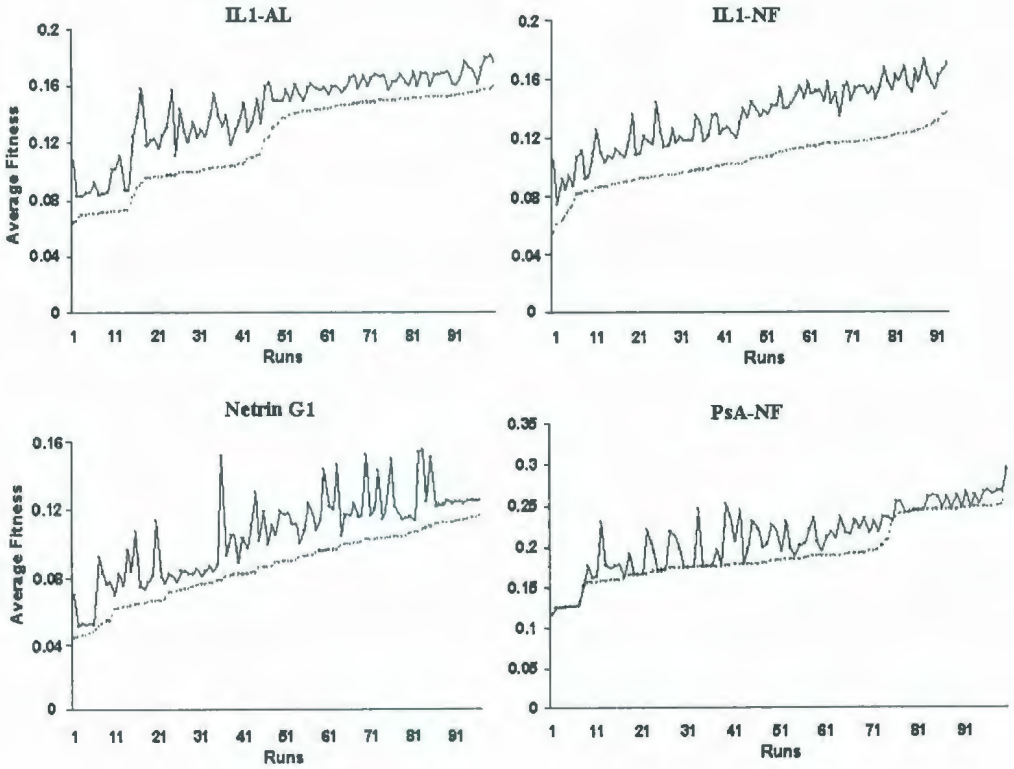
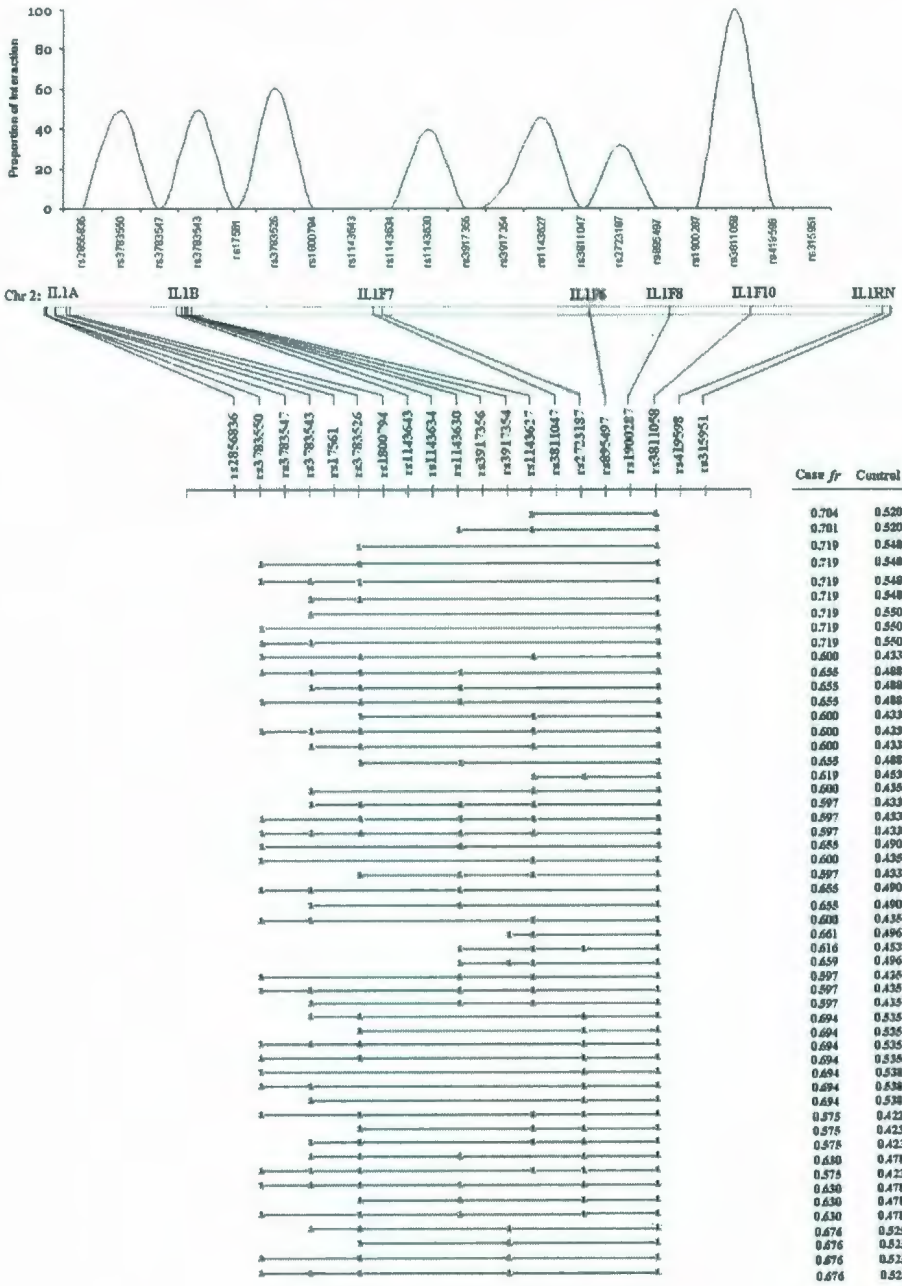


**Table A-1. Distribution characteristics and statistical significance tests for the performance of CCA on each dataset.**

The pairwise t-test was performed to quantify the level of significant difference among average fitness and average maximum fitness to ensure the evolvability of the CCA.

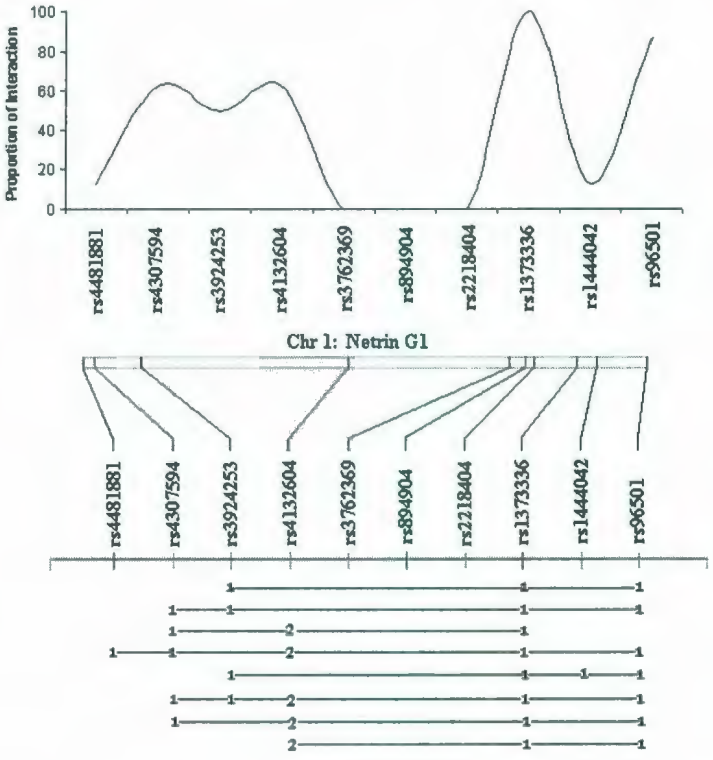
| | Average Fitness | | | Average Maximum Fitness | | | t-test |
|---|---|---|---|---|---|---|---|
| | Average | Std | Mean (95% CI) | Average | Std | Mean (95% CI) | |
| **IL1-AL** | 0.121 | 0.030 | 0.122(0.116-0.127) | 0.143 | 0.027 | 0.144(0.137-0.149) | $1.0 \times 10^{-4}$ |
| **IL1-NF** | 0.103 | 0.016 | 0.104(0.099-0.107) | 0.133 | 0.022 | 0.134(0.129-0.137) | $1.0 \times 10^{-4}$ |
| **Netrin G1** | 0.085 | 0.019 | 0.085(0.081-0.917) | 0.104 | 0.024 | 0.105(0.100-0.109) | $1.0 \times 10^{-4}$ |
| **PsA** | 0.191 | 0.036 | 0.192(0.184-0.199) | 0.210 | 0.040 | 0.211(0.202-0.218) | $7.0 \times 10^{-4}$ |

**Figure A-4: Results of the Competitive Co-Evolutionary Algorithm for the Il-1 gene cluster in AS**




| Case *fr* | Control *fr* | HRR (CI 95%) |
|---|---|---|
| 0.704 | 0.520 | 2.193(1.631-2.914) |
| 0.701 | 0.520 | 2.167(1.612-2.878) |
| 0.719 | 0.548 | 2.119(1.585-2.850) |
| 0.719 | 0.548 | 2.119(1.585-2.850) |
| 0.719 | 0.548 | 2.119(1.585-2.850) |
| 0.719 | 0.548 | 2.119(1.585-2.850) |
| 0.719 | 0.550 | 2.097(1.568-2.822) |
| 0.719 | 0.550 | 2.097(1.568-2.822) |
| 0.719 | 0.550 | 2.097(1.568-2.822) |
| 0.600 | 0.433 | 1.964(1.486-2.607) |
| 0.655 | 0.488 | 1.992(1.502-2.652) |
| 0.655 | 0.488 | 1.992(1.502-2.652) |
| 0.655 | 0.488 | 1.992(1.502-2.652) |
| 0.600 | 0.433 | 1.964(1.486-2.607) |
| 0.600 | 0.433 | 1.964(1.486-2.607) |
| 0.600 | 0.433 | 1.964(1.486-2.607) |
| 0.655 | 0.488 | 1.992(1.502-2.652) |
| 0.619 | 0.453 | 1.965(1.474-2.589) |
| 0.600 | 0.438 | 1.944(1.471-2.580) |
| 0.597 | 0.433 | 1.944(1.471-2.580) |
| 0.597 | 0.433 | 1.944(1.471-2.580) |
| 0.597 | 0.433 | 1.944(1.471-2.580) |
| 0.655 | 0.490 | 1.972(1.487-2.626) |
| 0.600 | 0.438 | 1.944(1.471-2.580) |
| 0.597 | 0.433 | 1.944(1.471-2.580) |
| 0.655 | 0.490 | 1.972(1.487-2.626) |
| 0.658 | 0.490 | 1.972(1.487-2.626) |
| 0.600 | 0.438 | 1.944(1.471-2.580) |
| 0.661 | 0.496 | 1.976(1.490-2.623) |
| 0.616 | 0.453 | 1.944(1.459-2.561) |
| 0.659 | 0.496 | 1.96(1.474-2.603) |
| 0.597 | 0.438 | 1.924(1.456-2.553) |
| 0.597 | 0.438 | 1.924(1.456-2.553) |
| 0.597 | 0.438 | 1.924(1.456-2.553) |
| 0.694 | 0.535 | 1.975(1.483-2.645) |
| 0.694 | 0.535 | 1.975(1.483-2.645) |
| 0.694 | 0.535 | 1.975(1.483-2.645) |
| 0.694 | 0.535 | 1.975(1.483-2.645) |
| 0.694 | 0.538 | 1.955(1.468-2.619) |
| 0.694 | 0.538 | 1.955(1.468-2.619) |
| 0.694 | 0.538 | 1.955(1.468-2.619) |
| 0.575 | 0.423 | 1.846(1.397-2.447) |
| 0.575 | 0.423 | 1.846(1.397-2.447) |
| 0.575 | 0.423 | 1.846(1.397-2.447) |
| 0.630 | 0.478 | 1.859(1.405-2.470) |
| 0.575 | 0.423 | 1.846(1.397-2.447) |
| 0.630 | 0.478 | 1.859(1.405-2.470) |
| 0.630 | 0.478 | 1.859(1.405-2.470) |
| 0.630 | 0.478 | 1.859(1.405-2.470) |
| 0.676 | 0.525 | 1.886(1.411-2.503) |
| 0.676 | 0.525 | 1.886(1.411-2.503) |
| 0.676 | 0.525 | 1.886(1.411-2.503) |
| 0.676 | 0.525 | 1.886(1.411-2.503) |

## Figure A-5: Results of the Competitive Co-Evolutionary Algorithm in Schizophrenia




| Case *fr* | Control *fr* | HRR (CI 95%) |
|---|---|---|
| 0.497 | 0.342 | 1.906(1.412-2.572) |
| 0.236 | 0.117 | 2.187(1.454-3.290) |
| 0.242 | 0.122 | 2.34(1.566-3.497) |
| 0.328 | 0.211 | 1.908(1.329-2.740) |
| 0.272 | 0.164 | 1.822(1.304-2.546) |
| 0.222 | 0.114 | 2.289(1.541-3.399) |
| 0.219 | 0.114 | 2.223(1.479-3.341) |
| 0.264 | 0.161 | 1.867(1.296-2.688) |